A Mutagenic study of functional and structural aspects of rat Insulin-like Growth Factor Binding Protein - 5

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Thesis submitted to the University of Glasgow in accordance with the requirements for the degree of Doctor of Philosophy in the Faculty of Science

Hannah Research Institute, Ayr
Feburary 2001

DECLARATION

The work contained in this thesis was carried out by myself at the Hannah Research institute, under the supervision of Dr. G. Allan and Dr. J. Beattie. No part of this work has been submitted for consideration for any other degree or award.

Hyuk Song

Abstract

On going research in our laboratory is focussed on the potential role of Insulin-like growth factor binding protein-5 (IGFBP-5) in apoptosis of mammary epithelial cells. In order to increase our understanding of IGFBP-5 functions, the work described below focussed on the relationship between the structure of the binding protein and its important molecular interactions.

Using site-directed mutagenesis, we have mutated two highly conserved amino acids, Gly203 and Gln209 (G203K and Q209A respectively) within the basic amino acid rich region (201-218) in the C-terminal domain of rat IGFBP-5. After analysis of binding activity using three different methods – IGF ligand blotting, IGF solution phase equilibrium binding and biosensor measurement – we have shown that the mutation of either of these two residues results in a reduction in affinity of the binding protein for both IGF-I and IGF-II by approximately ten-fold. Furthermore, mutation of both amino acids (G203K/Q209A, termed the Double mutant) had a cumulative effect and results in the complete ablation of IGF-I binding and a several thousand-fold reduction in IGF-II binding. This reduction in IGF binding affinity was comparable to that observed for the C-terminally truncated mutant BP550 (residues 1-168), which suggests that Gly203 and Gln209 may be the major determinants for IGF binding in the C-terminal domain. In addition, using heparin ligand blots, we confirm that mutation of basic residues within the C-terminal 201-218 region (Hep- mutant: R201L, K202E, K206Q and R214A) results in major attenuation of heparin binding, whereas the G203K and Q209A single mutants and the Double mutant have no reduction in heparin binding. Therefore, our data suggests a potential overlap of heparin- and IGF-binding domains in the C-terminal region of IGFBP-5, and based on this, we discuss a potential model to explain the observed lower IGF binding affinity of ECM-bound IGFBP-5.

In an attempt to assess the separate contribution of the N- and C-terminal domains to IGF binding, we also made four chimeric IGFBP cDNAs, BP552, BP522, BP255 and BP225, by switching domains between rat IGFBP-5 and rat IGFBP-2. We were consistently unable to detect expression of BP225 protein in the baculovirus/insect cell system, so this chimera had to be excluded from further study. Recognition of BP552 and BP522 proteins by both anti-rat IGFBP-5 and anti-rat IGFBP-2 antisera confirms their identity as chimeric proteins made up of domains from

the native binding proteins, while BP255 was only recognised by the anti-rat IGFBP-5. The IGF binding properties of unpurified BP552, BP522 and BP255 proteins were assessed by IGF ligand blotting and IGF solution phase binding assays. These experiments demonstrated that BP552 and BP522 had comparable affinities with native IGFBP, whereas we were unable to detect any binding of BP255 to IGF-I by either technique, and only very weak interaction with IGF-II. Purified BP552 and BP522 were also tested by biosensor analysis, and data confirmed the results from the previous experiments. From this we can conclude that the N-terminal domain of IGFBP-5 can co-operate with the C-terminal domain of IGFBP-2 to produce a high affinity IGF binding species, irrespective of which central domain is present, whereas preliminary evidence would suggest that this co-operation may not occur between the N-terminal domain of IGFBP-2 and the C-terminal domain of IGFBP-5.

In addition to IGF binding, the IGFBP-5 protein contains consensus heparin binding motifs in both its C-terminal and central domains, although only the C-terminal site was previously shown to be functional. Using heparin ligand blotting, it was found that the ability of BP552 and BP550 to bind to heparin was equivalent to that of WTIGFBP-5, whereas WTIGFBP-2 and BP522 failed to bind. These results demonstrate that an active heparin binding site in BP552 and BP550 is contained within the central domain of IGFBP-5, and that this site is only active in the absence of the carboxy-terminal domain. We subsequently mutated two basic amino acids (R136A:R137A) in the central consensus binding sites between residues 132-140. This resulted in the loss of heparin binding for BP550, confirming the importance of these two basic amino acids in the central domain heparin binding activity. In light of these findings, we suggest that C-terminally truncated fragments of IGFBP-5 generated *in vivo* by proteolysis could retain heparin/extracellular matrix (ECM) binding properties.

Finally, we propose that our various IGFBP-5 mutants described above provide an opportunity to test the structure-function relationships of this binding protein in an appropriate biological context.

Acknowledgements

The work described in this thesis was carried out in the Molecular Recognition group at the Hannah Research Institute, Ayr, UK and all my three years tuition fees and living expenditures were supported by British Council, Manchester, UK and this institute.

I would particularly like to thank my great supervisor Dr Gordon Allan for his continuous support and encouragement throughout this project. To be honest, before I met him, I didn't have any molecular biological experience, in a word - I was nothing for science. In spite of the extremely hard supervision required for me, he has continuously motivated and taught me to be a good scientist. I am also extremely lucky to have had another supervisor, Dr James Beattie, who also supported and encouraged me throughout my Ph.D course.

I would also like to thank Prof. Kil Saeng Choung and Prof. Hoon Taek Lee of Kon-Kuk Universtiy in Korea for their encouragement to start and complete this challenging work, so far from home SOB SOB. My thanks also to Drs David Flint, John Shand and Elizabeth Tonner helping me many times with lab techniques and analysing and discussing my results. A big thanks to Silvia Bramani for her personal friendship and encouragement.

Finally, I would like to give my big appreciation to my wonderful parent, brother and other family, specially uncle Ki-ju and anty, whose support and affection was invaluable to me in these three years abroad. Of course, I cannot forget to mention my wife, Una for her continuous support and trust. I wish this thesis would be a good present for my unborn baby, Sat Byol.

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List of Abbreviation

ALS	Acid labile subunit
Con	Concentrated protein preparation
Cpm	Counts per minute
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
GAGs	Glycosaminoglycans
HOX	Homeobox gene
HRP	Horse radish peroxidase
IGFBP	Insulin-like growth factor binding protein
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor-II
IGF-II-R	type II IGF receptor
IGF-I-R	type I IGF receptor
kDa	kilo Dalton
Mr	Molecular weight
Wt	Wild type

Chapter I. Introduction

I.1 Overview of the IGF axis

The insulin-like growth factors (IGFs), IGF-I and IGF-II, are small single chain peptides that share significant structural homology with insulin. They are involved in the regulation of cell growth, differentiation, and metabolism, eliciting diverse effects in multiple biological processes (reviewed by Stewart and Rotwein, 1996a). The biological effects of IGFs are mediated through binding to high-affinity receptors on the cell surface. The type I IGF receptor (IGF-I-R) is, like the insulin receptor, a heterotetrameric protein complex containing a tyrosine kinase domain that mediates signal transduction (De meyts *et al*, 1994). Ligand binding to this receptor results in growth and differentiative functions (Cheatham and Kahn, 1995; Le Roith *et al*, 1995), and its inactivation in receptor null mice leads invariably to severe neonatal growth deficiencies as a consequence of hypoplasia in several tissues (Liu *et al*, 1993a). The structurally distinct type II IGF receptor (IGF-II-R) is a monomeric protein lacking tyrosine kinase activity, and is also known as the cation dependent mannose-6-phosphate receptor. There is no known signal transduction mechanism initiated by this receptor, so that all proliferative effects of the IGFs are thought to be mediated through the IGF-I-R (Czech, 1989). However, the IGF-II-R appears to play an important role in the degradation of IGF-II, and inactivation of this receptor in mice by gene targeting results in foetal overgrowth, skeletal abnormalities, and perinatal death due to overexposure of the foetus to IGF-II (Wang *et al*, 1994; Lau *et al*, 1994; Ludwig *et al*, 1996).

Unlike insulin, the IGFs are bound in serum and other biological fluids to a family of six structurally and evolutionary related binding proteins, termed IGF-binding proteins (IGFBP-1 to IGFBP-6). In the circulation, ~75-80% of the IGFs are present in a complex of molecular weight 150kDa, which is composed of one molecule of IGF-I or IGF-II, an 85kDa ALS (termed the acid labile subunit - a liver derived glycoprotein) and either IGFBP-3 or -5. A smaller proportion (20-25%) of the IGFs are associated with other IGFBPs forming a 50kDa complex, and less than 1% is found in the free form of ~7.5kDa (Rajaram *et al*, 1997).

The IGFBPs are expressed in a tissue-specific manner and have different affinities for the IGFs (reviewed in Clemmons, 1997). In addition to their role in preventing insulin-like side effects of the relatively large concentrations of IGFs in the circulation, the IGFBPs are also able to modulate different IGF actions positively or negatively by interruption of the IGF ligand-receptor interaction. Including at least four other low-affinity IGF binders, termed insulin-like growth factor binding protein-related proteins (IGFBP-rP) these proteins are part of a superfamily (Baxter *et al*, 1998). The IGFBPs are able to modulate the action of IGFs due to the fact that they have at least 50-fold higher affinity for binding IGFs than the IGF-I-R. However, at least three mechanisms have been shown to alter the affinity of the IGFBPs, making the network of regulatory components in the IGF system even more complex: IGFBP proteolysis, phosphorylation, and adherence to the cell surface or to the extracellular matrix (ECM) (Clemmons, 1997).

Most of the IGFBPs bind IGF-I and IGF-II with more or less equal affinity except for IGFBP-2 and –6, which have higher affinities for IGF-II (Kiefer *et al*, 1991). In plasma, the IGFs bind to IGFBPs, which control the bioavailability of circulating IGFs by prolonging their half-lives as well as delivering them to their target tissues. At the tissue or cellular level IGFBPs may exist in two compartments; either in the interstitial compartment or cell culture medium where the IGFBPs are in the soluble form, or bound to ECM and/or cell membranes. Soluble and immobilised IGF-IGFBP complexes could potentially prevent the IGFs from interacting with the receptor, thereby inhibiting the biological effects of the IGFs. This effect has been demonstrated for all six IGFBPs in diverse biological systems (Cohick and Clemmons, 1993; Rechler, 1993).

Binding of IGFs requires an intact binding protein, and proteolytic cleavage of the IGFBPs generates fragments that have greatly reduced affinity for IGFs. Thus, proteases may function to release free IGFs, which are then able to bind to receptors. Furthermore, glycosaminoglycans such as heparin, heparan sulphate and dermatan sulphate regulate the interactions of IGFBP-3 and -5 with IGF-I and inhibit proteolysis of the IGFBPs. Thus, these proteoglycans represent a further level of control of the IGF-IGFBP complexes and IGF action (Arai *et al*, 1994). Finally, the IGFBPs may have intrinsic cellular functions in the absence of IGFs, suggesting that they may act via their own receptor (Oh *et al*, 1993b, c). These relationships are summarised in Figure I.1.

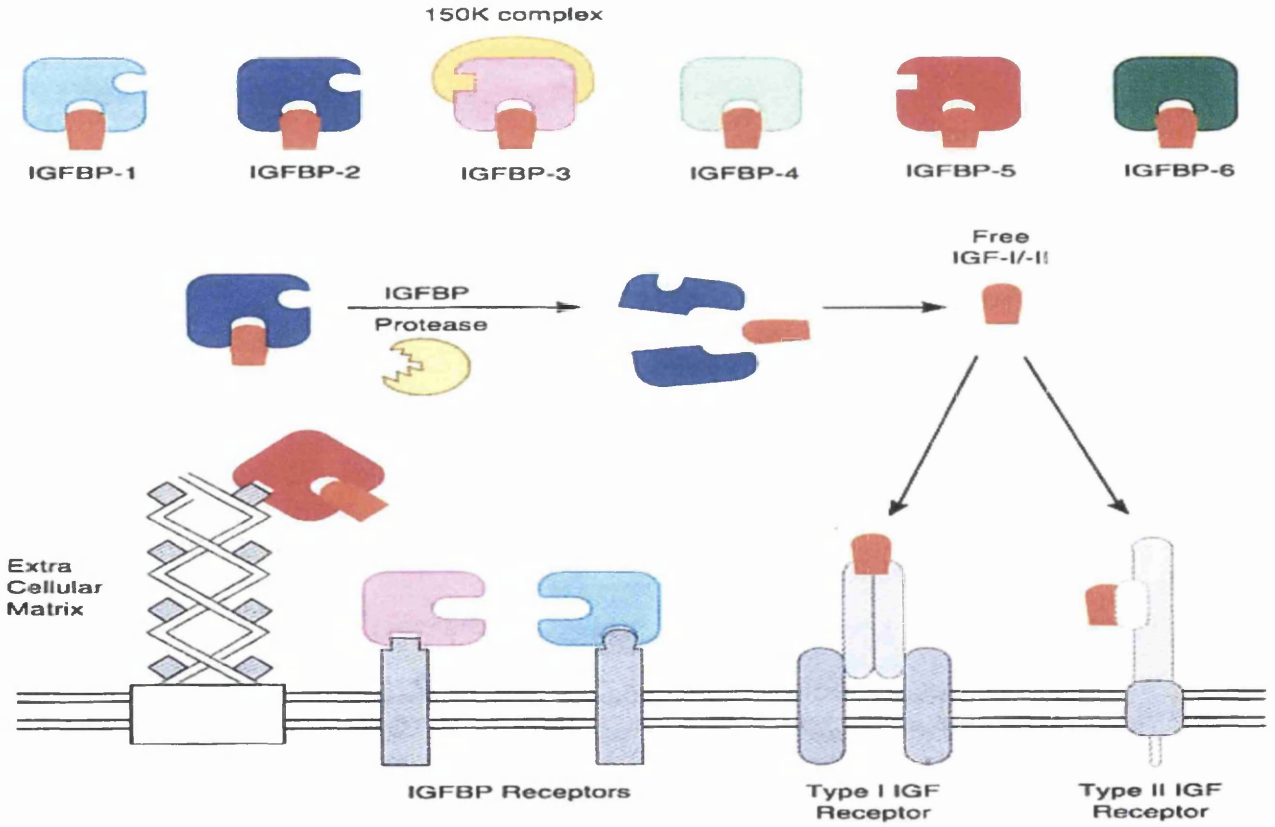


Figure I.1 Overview of IGF/IGFBP system

I.2 Insulin-like growth factors (IGFs)

The term Insulin-like growth factor was proposed in 1976 (Rinderknecht and Humble, 1976) to signify the relationship of these substances to insulin and to emphasise their growth promoting activities. IGF-I is a single-chain basic protein of 70 amino acids, whereas IGF-II is a slightly acidic single-chain peptide of 67 residues (Rinderknecht and Humble, 1976, 1978). Both IGF-I and -II show ~70% homology to each other, and their A and B domains show ~50% homology to A and B chains of human insulin (Phillips *et al*, 1998). The B domain contains the N-terminus of the IGF protein and the D domain represents the C-terminus, whereas, domain C links the A and B domain.

I.2.1. IGF Receptors

IGFs interact with specific receptors designated type I (IGF-I-R) and type II (IGF-II-R) IGF receptors, as well as with the insulin receptor. Most of the mitogenic effects of the IGFs are considered to be mediated via IGF-I-R.

I.2.1.1 IGF-I-R

The IGF-I-R is a disulphide-linked heterotetrameric glycoprotein composed of two ligand-binding α-subunits of 706 amino acid and two transmembrane β-subunits of 627 residues. The human protein is produced by mRNAs derived from the single 21-exon IGF-I-R gene, located on chromosome 15q25-q26 (Abbot *et al*, 1992; Ullrich *et al*, 1986).

The α-subunits contain the ligand-binding region of the receptor. The IGF-I-R binds IGF-I with a dissociation constant (Kd) of ~ 1nM in intact cells; IGF-II binds with several-fold lower affinity, and insulin with more than 100-fold lower affinity (Jones and Clemmons, 1995; Le Roith *et al*, 1995). The β-subunits are composed of a short extracellular domain, a membrane-spanning segment, and a large intracytoplasmic region containing a tyrosine kinase domain and sites of tyrosine and serine phosphorylation (Le Roith *et al*, 1995; Ullrich *et al*, 1986). Ligand binding to the α-subunits triggers activation of the intracellular tyrosine kinase, leading to receptor autophosphorylation by an intramolecular trans-phosphorylation mechanism similar to that used by

other receptor tyrosine kinases (Frattali and Pessin, 1993; Jones and Clemmons, 1995; LeRoith *et al*, 1995). The autophosphorylation activates the inherent tyrosine kinase activity of the receptor, that leads to phosphorylation of other important tyrosines on the receptor as well as on endogenous substrates (Kato *et al*, 1992).

The IGF-I receptor is ubiquitously expressed, with highest levels seen during embryonic development and organogenesis. Mice homozygous for a null mutation of this receptor are small in size and die at birth. Their inability to develop properly is largely due to respiratory failure associated with underdeveloped respiratory muscles as well as poorly developed lungs and brains, and decreased bone ossification (Liu *et al*, 1993a; Baker *et al*, 1993).

I.2.1.2 IGF-II/Mannose-6-phosphate (M-6-P) receptor

The IGF-II-R is a single-chain membrane-spanning glycoprotein that also is known as the cation-independent mannose-6-phosphate receptor. The mature human receptor contains 2,451 amino acids that can be divided into three regions, a large 2,264-residue extracellular domain, a 23-amino acid transmembrane region, and a 164-residue carboxyl-terminal cytoplasmic domain (Morgan *et al*, 1987; Oshima *et al*, 1988). The extracellular part of the IGF-II-R binds ligand, and the cytoplasmic region encodes segments responsible for sorting among different subcellular compartments (Rohrer et al, 1995; Lobel *et al*, 1988), for endocytosis (Lobel *et al*, 1988), and potentially for coupling to inhibitory GTP-binding proteins (Nishimoto *et al*, 1989; Okamoto *et al*, 1990). The IGF-II-R is highly conserved among different species, with ~80% homology being found among bovine, rat, mouse, and human receptors (Kornfeld, 1992). The extracellular domain of each receptor is composed of 15 contiguous segments of 134-191 residues repeats that share 16-38% identity (Lobel *et al*, 1988; Macdonald *et al*, 1988; Morgan *et al*, 1987; Szebenyi and Rotwein, 1994; Zhou and Sly, 1995). As mentioned before, no known IGF effects have been shown to be mediated by the IGF-II-R.

1.2.2. Functions of IGFs

The *in vivo* effects of IGFs are mainly anabolic insulin-like effects (stimulation of glucose uptake and glycogen synthesis) in fat and muscle cells (Froesch *et al.*, 1985), and a growth promoting effect through mediation of GH (growth hormone) effects (Salmon and DuVall, 1970). Stimulation of cell proliferation in a variety of organs and tissues by IGFs has also been observed including nervous system development (Heidenreich, 1993), osteoblasts (Hock *et al*, 1988), bone endothelial cells (Fiorelli *et al*, 1994), chondrocytes (Ohlsson *et al*, 1992), hormone synthesis in ovary (Davoren *et al*, 1985; Talavera and Menon, 1991; Hernandez *et al*, 1988; Christman *et al*, 1991; Erickson *et al*, 1991), spermatogonial cells in testes (Soder *et al*, 1992), and various cancer cells (reviewed by Le Roith, 1995).

In vitro, IGFs promote cell cycle progression from the Go\G1 to the S phase, resulting in DNA synthesis and cell proliferation (reviewed by Le Roith and Roberts, 1993). IGFs stimulate a mitogenic response in many cell types whereas in haematopoietic cells and in some carcinoma cell lines they can function as survival factors preventing apoptosis (reviewed by Le Roith and Roberts, 1993). Proliferation and apoptosis signals may share some common pathways as they both induce similar changes in the morphology of the cells (chromatin condensation, loss of cell-cell contact inhibition, nuclear disintegration). This is supported by the observation that overexpression of proteins is normally associated with cell proliferation (Hartwell and Kastan, 1994; Steller, 1995; Askew *et al.*, 1991; White *et al.*, 1991). However, IGF-I has been shown to support viability in non proliferating cells in culture (Carlsson-Skwirut *et al.*, 1989; Beck, 1994; Bozyczko-Coyne *et al.*, 1993; LeRoith *et al.*, 1993; Svrzic and Schubert, 1990) suggesting that stimulation of cell proliferation and anti apoptotic action may be two distinct signals (Rubin and Baserga, 1995).

Secondly, IGFs stimulate cell differentiation in osteoblasts (Hall and Chambers, 1990), chondrocytes (Geduspan and Solursh, 1993), adipocytes and neural cells (Pahlman *et al.*, 1991), and induce an acute insulin-like effect on protein and carbohydrate metabolism, especially in myoblasts (Dimitriadis *et al.*, 1992). They also regulate the hormone secretion or induction of chemiotactic migration in specific cell types (Stracke *et al.* 1989; El-Badry *et al*, 1990).

I.3 Insulin-like growth factor binding proteins (IGFBPs)

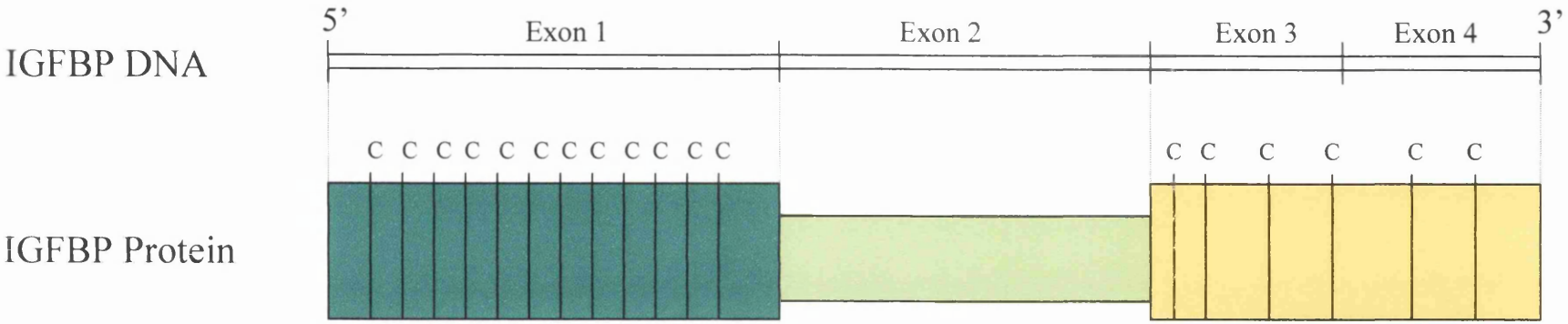
IGFBPs are produced by a variety of different tissues, and each tissue has specific patterns of IGFBP secretion. The six proteins also differ significantly in their biochemical characteristics, and this accounts for many of the differences that have been observed in their biological actions. In addition, proteolysis of IGFBPs by proteases plays a key role in modulating levels and actions of free IGFs and IGFBPs, and four IGFBP related proteins have been discovered recently which can also affect IGF action (Hwa *et al*, 1999).

1.3.1 General structure of IGFBPs

IGFBPs share at least 50% homology among themselves, and 80% homology between different species (Lamson *et al*, 1991; Shimasaki and Ling, 1991). Figure I.2 shows the structure of IGFBP, where the molecule can be divided into three structurally distinct domains. Most of the homology is conserved in the N- and C- terminal domains, whereas the middle central domain has little similarity among the different IGFBPs (Hwa *et al*, 1999). Each protein contains a leader (signal) sequence with a hydrophobic domain, and all are known to be synthesized and secreted by at least one cell type. The proteins vary in length from 216 (IGFBP-6) to 289 amino acids (IGFBP-2). All six proteins contain 18 cysteines that are conserved (IGFBP-4 contains two additional cysteines in the central domain).

IGFBP-5 is produced as a 252 amino acid protein of ~29kDa. cDNA clones have been isolated and sequenced from human, rat, mouse, and chicken (Kiefer *et al*, 1991; Shimasaki *et al*, 1991; Zhu *et al*, 1993; Allander *et al*, 1997). A 6.0 kb IGFBP-5 mRNA is expressed in a large variety of tissues and cell types, showing greatest abundance in the kidney. In some cell types smaller transcripts have been reported (Cohen *et al*, 1994).

The highly conserved amino- (N-) and carboxy- (C-) terminal domains are cysteine rich with 12 cysteines in the N-terminus and 6 in the C-terminus. The high number of cysteine residues in these domains suggest that these parts of the IGFBP proteins are highly structured, and therefore their three dimensional structure might be an important factor in their function. It is believed all of the cysteines are engaged in disulphide bridges, and disulphide linkages of cysteines are important



N-and C-terminal domain: Cysteine rich
Sequence homology between IGFBPs
Bind IGFs.

Central Domain : No cysteines (IGFBP 4 has 2)
No sequence homology between IGFBPs

Figure I.2 IGFBPs contain three structurally distinct domains

for the correct folding and maintenance of the three-dimensional structure of many proteins. In addition it is believed that intradomain disulphide bond formation is more likely than interdomain disulphide linkages between cysteines in the N- and C-terminal domains.

Recently, the three C-terminal disulphide linkages of bovine IGFBP-2 were determined to be Cys^{13}-Cys^{14}, Cys^{15}-Cys^{16}, and Cys^{17}-Cys^{18} (Forbes *et al*, 1998). In addition, cyanogen bromide cleavage of this protein revealed that the N- and C-terminal domains were not linked by disulphide bonds (Forbes *et al*, 1998). The C-terminal disulphide linkages of human IGFBP-6 were also partially determined and are consistent with those of IGFBP-2 (Neuman *et al*, 1998). A structural study of IGFBP-5 has shown that the disulphide-linkages in N-terminal domain of human IGFBP-5 are Cys^{1}-Cys^{2}, Cys^{3}-Cys^{4}, Cys^{5}-Cys^{6}, Cys^{7}-Cys^{9}, and Cys^{8}-Cys^{10} (Neuman *et al*, 1999). Although, the disulphide linkages of cysteines 11 and 12 were not determined, inter-domain links with cysteines in the C-terminal domain of IGFBP-5 cannot be ruled out at present. This pattern of disulphide-linkages of the N-terminal domain of IGFBP-5 is identical to that of IGFBP-3 (Hashimoto *et al*, 1997; Kalus *et al*, 1998). IGFBP-6 also showed the same pattern of disulphide-linkages in the N-terminal domain, although, partial determination of the N-terminal domain disulphide-linkages of IGFBP-1 showed a different pattern, where Cys^{1} is not linked to Cys^{2} and Cys^{3} is not linked to Cys^{4} (Neumann *et al*, 1999).

In contrast to the N- and C-terminal domains, the central domain has no sequence homology between IGFBPs and also no cysteines. However, this region contains phosphorylation, glycosylation, and many proteolytic cleavage sites suggesting that important biological functions of IGFBPs are regulated within this region (Conover, 1995).

The IGFBP superfamily also encompasses several IGFBP-related proteins (IGFBP-rPs) which bind IGFs with low affinity (Baxter *et al*, 1998). IGFBPs and IGFBP-rPs share the highly conserved and cysteine-rich N-terminal domain which appears to be crucial for several biological actions, including their binding to IGFs (Yamanaka *et al*, 1997; Vorwerk *et al*, 1997).

I.3.2 Evolution of IGFBP gene

While the six IGFBPs clearly have distinct amino acid sequences, they do share regions of strong homology and a similar genomic organisation (Allander *et al*, 1994; Binkert *et al*, 1992; Gao

et al, 1993; Kou *et al*, 1994a-b; Upton *et al*, 1993; Zhu *et al*, 1993), and the evolutionary conservation of IGFBPs supports their fundamental importance in regulatory processes. All human IGFBP genes lie in close proximity to homeobox (HOX) gene clusters on the different chromosomes, implying a genetic link between these two gene families. The vertebrate homeobox (HOX) genes are suggested to be true homologs to the insect homeotic gene complexes and to have arisen from duplications of a single ancestral gene cluster (Akam, 1989; Schugart *et al*, 1989; Pendleton *et al*, 1993). The human HOX gene family consists of four gene clusters, HOX A-D, localized to chromosomes 2, 7, 12, and 17 respectively (Scott, 1992; Cannizzaro *et al*, 1987; Acampora *et al*, 1989). As shown in Figure I.3, the HOX and IGFBP genes/clusters are localized to the same chromosomal regions. Furthermore, the human IGFBP-2/-5 and IGFBP-1/-3 gene pairs are closely linked (Allander *et al*, 1994). Similarly, the two mouse IGFBP gene pairs (IGFBP-2/-5 and IGFBP-1/-3) are also closely linked (Kou *et al*, 1994b).

In functional and structural aspects, IGFBP-3 and –5 are more closely related to each other than to other IGFBPs, and this is also true for IGFBP-1 and –2. It has been suggested that all 6 IGFBP genes have evolved from a single ancestral IGFBP gene through a process of gene duplication and chromosomal translocation and were dispersed to multiple chromosomal loci. In this model, IGFBP-3 and –5 were duplicated from a single gene, as were IGFBP-1 and –2. Thus, the evolution of the HOX and IGFBP gene families appears to follow a similar pattern (Allander *et al*, 1995), and the association of the human genes at the same chromosomal loci suggests that ancesteral IGFBP and HOX genes were linked before the first duplication of chromosomal DNA containing the ancestral HOX cluster (Allander *et al*, 1994). Whether there is any functional significance to this linkage remains to be seen. It should be noted that the mouse IGFBP genes do not share the same chromosomal localization as mouse HOX A and D (Kou *et al*, 1994a), and this might reflect two different evolutionary pathways after the divergence of humans and mice (Allander *et al*, 1995).

I.3.3 IGF binding motifs

Although the structure of IGFBP proteins has not as yet been determined by X-ray crystallography, it is believed that the approximately 30kDa IGFBP proteins make contact with the

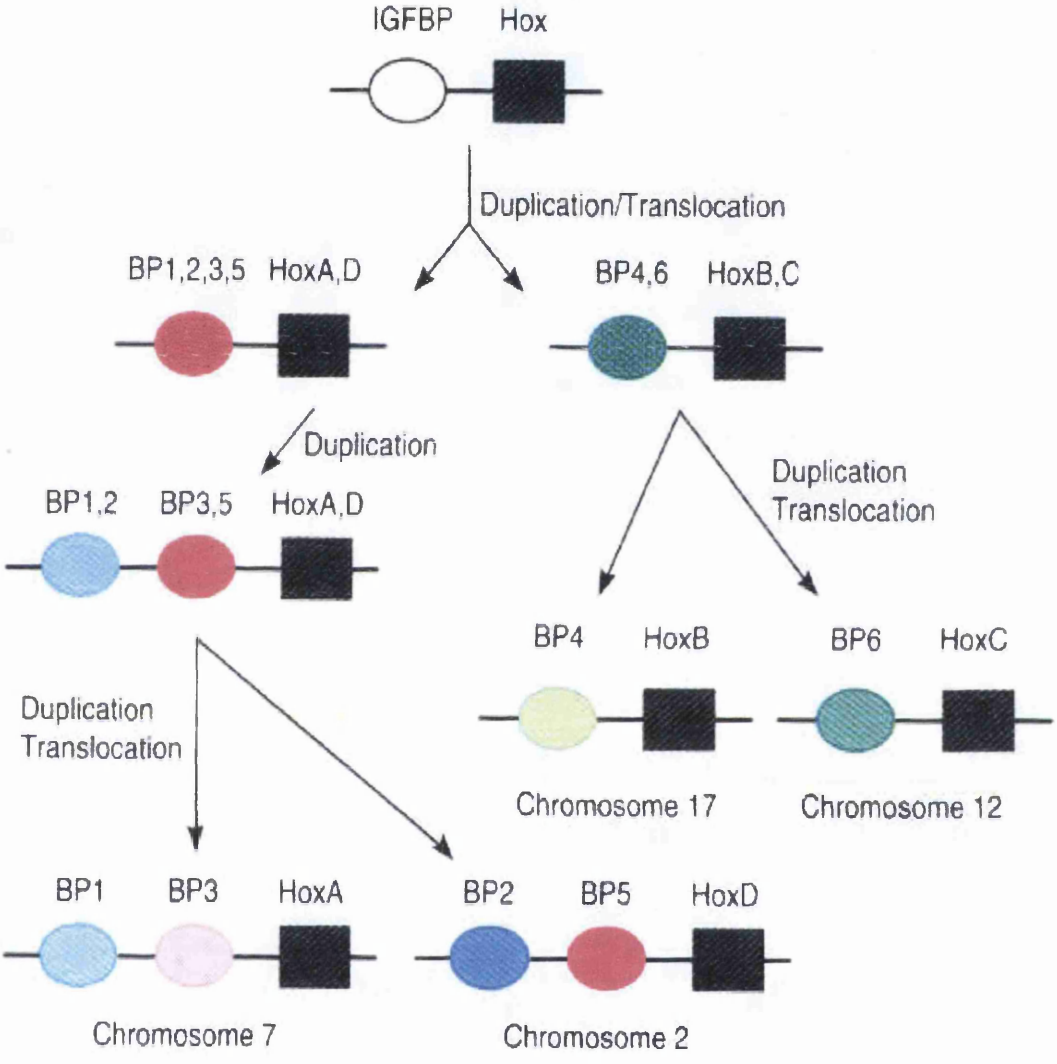


Figure I.3 Schematic diagram of IGFBP/Hox gene evolution

much smaller IGF molecules (approximately 7.5kDa) with their N- and C-terminal domain simultaneously. Thus, the general structure of the IGFBP protein may be envisioned as two highly folded terminal domains in close contact, connected by a flexible central domain. Supporting this hypothesis, *in vivo* observations have initially shown that in biological fluids, IGFBPs can be proteolysed to fragments with diminished affinities for IGFs. To date, many researchers have performed experiments aimed at identifying the IGF binding motifs in N- and C-terminal domain of IGFBPs.

N-terminal domain Studies on human IGFBP-4 showed that the sequence Leu72-Ser91 is important for IGF-II binding, as deletion of this region resulted in a protein which was undetectable by ligand blot (Qin *et al*, 1998). In support of this finding, it was shown that residues between Glu^{52}-Ala^{92} in the N-terminal domain of rat IGFBP-3 are critical for binding IGF-II (Hashimoto *et al*, 1997). Recently, work from several laboratories has begun to identify the specific amino acids in the IGFBP molecule that contribute to IGF binding. At the N-terminus of bovine IGFBP-2, it was shown that Tyr60 was protected against iodination upon binding of IGFBP-2 to IGF-II (Hobba *et al*, 1996). Subsequently, the same group demonstrated that substitution of Tyr60 with Ala or Phe leads to a 4.0- and 8.4-fold reduced affinity for IGF-I respectively, and a 3.5- and 4.0-fold reduced affinity for IGF-II respectively (Hobba *et al*, 1998). These researchers suggested that the hydroxyl group of Tyr60 may participate in hydrogen bond formation and that this is important for the initial complex formation with IGF-I. They also suggested that this may be a common mechanism for all the IGFBPs, (with the exception of IGFBP-1 which has an alanine residue at the equivalent position). In support of this finding, Kalus *et al.* (1998) carried out nuclear magentic resonance spectroscopy (NMR) on a bacterially expressed N-terminal fragment of IGFBP-5 (residues 40-92). From this work it was proposed that the primary IGF binding sites comprise Val49, Tyr50 (equivalent to Tyr60 in IGFBP-2), Pro62 and Lys68 to Leu75, where the conserved Leu and Val residues localize in a hydrophobic patch on the surface of the IGFBP-5 protein (Kalus *et al*, 1998). Based on this study, Imai *et al.* (2000) mutated several amino acids within this hydrophobic patch between residues Val49 and Leu74 in both IGFBP-3 and –5. They found that the substitutions Lys68Asn, Pro69Gln, Leu70Gln, Leu73Gln, and Leu74Gln in IGFBP-5 (changing one charged residue, Lys68, to a neutral one and the four hydrophobic residues to

nonhydrophobic residues) resulted in an ~1000-fold reduction in the affinity of IGFBP-5 for IGF-I. Substitutions for homologous residues in IGFBP-3 (Arg69Ser, Pro70Ala, Leu71Ser, Leu74Gln, and Leu75Gly) also resulted in a >1000-fold reduction in affinity. Furthermore, they demonstrated that the physiological consequence of this reduction in affinity was that the mutated IGFBP-3 and -5 molecules became weak inhibitors of IGF-I-stimulated cell migration and DNA synthesis. Likewise, the ability of IGFBP-5 to inhibit IGF-I-stimulated receptor phosphorylation was attenuated. In conclusion, this study showed residues 68, 69, 70, 73, and 74 in IGFBP-5, as well as the homologous residues in IGFBP-3, appear to be critical for high affinity binding to IGF-I. Therefore, it was suggested that these residues form a binding pocket in both IGFBP proteins and were an important component of the core binding site (Imai *et al*, 2000). However, these researchers also demonstrated that the N-terminal fragments of IGFBP-5 have a 10- to 200-fold lower affinity for IGFs than the full length protein, which agrees well with the reported reduced affinities of other C-terminally truncated fragments of IGFBP-3 (Clemmons, 1993) and IGFBP-5 (Andress *et al*, 1993). This indicates that other residues at the C-terminus of the IGF binding proteins may be involved in the additional stabilisation of the IGFBP/IGF complex that leads to high affinity IGF binding.

C-terminal domain Mutagenesis of the carboxy end of IGFBP-1 showed that deletion of the C-terminal 20 amino acids resulted in loss of IGF binding as measured by ligand blotting (Brinkman *et al*, 1991). In contrast to IGFBP-1, deletion of a similar region in human IGFBP-4 (Lys215-Glu237) had no effect on IGF binding as determined by ligand blotting (Qin *et al*, 1998). Deletion of a further 10 amino acids (Cys205-Val214) reduced the binding to less than 15% of wild type IGFBP-4. However, it is conceivable that deletion of one of the very highly conserved cysteines in this region could have led to a major change in the secondary structure of the C-terminal domain, as the six cysteines at the C-terminus of bovine IGFBP-2 have been shown by Forbes *et al.* (1998) to form disulphide bonds between consecutive residues. In the Forbes study, the effects of four C-terminally truncated bovine IGFBP-2 mutants on IGF binding was also described (Figure I.4). Interestingly, the truncation of 48 residues from the C-terminus of bovine IGFBP-2 had no effect on IGF binding (equivalent to residue 200 in human IGFBP-4), suggesting that residues which contribute to IGF binding differ between IGFBP-2 and –4. However, greater

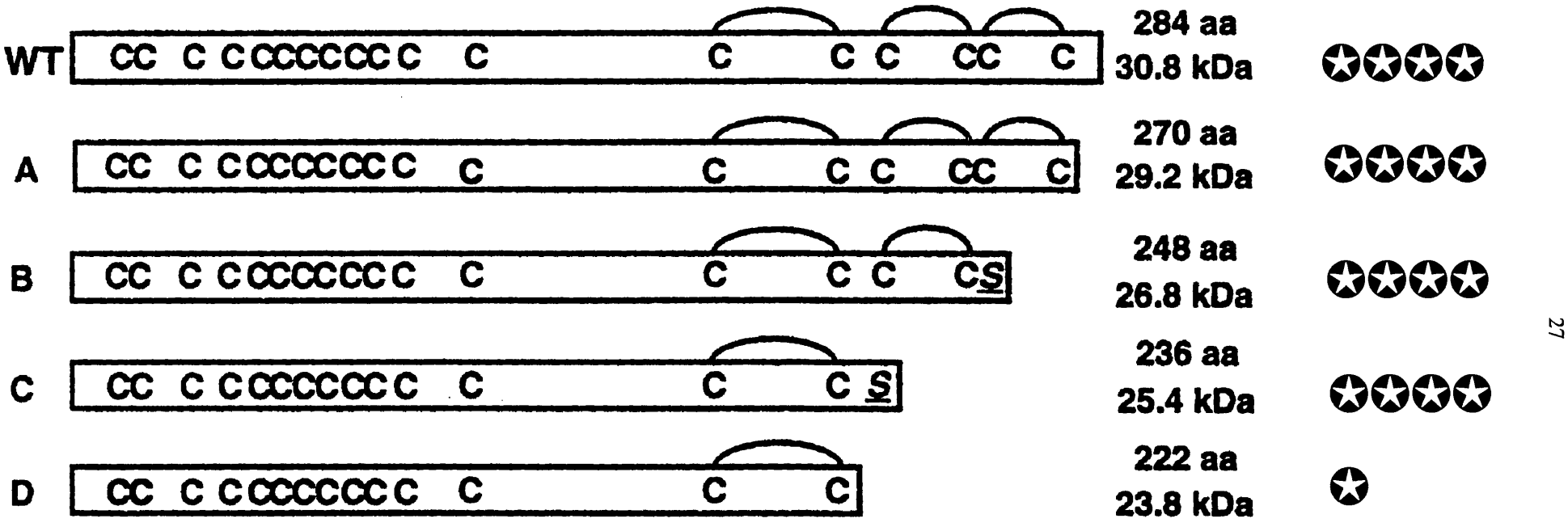


Figure I.4 Schematic outline of bovine IGFBP-2 and C-terminal deletion mutants.Bovine IGFBP-2 (WT) and mutants A-D are shown as boxes with N-terminal and C-terminal Cys (C) residues indicated and the C-terminal disulphide pattern highlighted. The peptide masses and residue length are shown, whereas Ser residues substituting for Cys are underlined. **(Forbes *et al* 1998 *JBC* 273: 4647-4652)** ✪ represents binding affinity to ^{125}I-IGF-II

reduction in affinity for both IGFs was observed when 63 residues were deleted from the C-terminus of IGFBP-2 (mutant D in Figure I.4), with a greater effect on IGF-II binding (up to 80-fold) as measured by charcoal binding solution phase assays. Therefore, this identified a critical region between amino acids 222-236 of bovine IGFBP-2 that is involved in binding IGFs (Forbes *et al*, 1998). These authors claimed that there was no real sequence conservation within this critical IGF binding region. Nevertheless, we decided to align the sequences from all six binding proteins from all species sequenced so far, and found two completely conserved amino acids in this region. Mutagenesis of these residues in IGFBP-5 and analysis of these effects on IGF and heparin binding forms the basis for Chapter III, and resulted in the publications Bramani *et al*, 1999 and Song *et al*, 2000.

The central domain of the IGFBPs has not been reported to contain any IGF binding properties. However, replacement of the IGFBP-3 central domain with that from IGFBP-2 resulted in an IGFBP chimera with reduced affinity for IGF-II (by at least 37%), which may suggest that the exact size of the central domain in each IGFBP may maximise high-affinity IGF binding by N-and C-terminal domains (Hashimoto *et al*, 1997).

I.3.4 Cell membrane and ECM binding motifs

Some IGFBPs not only bind IGFs, but also interact with cell surface molecules. IGFBP-1and -2 bind to the $\alpha_5\beta_1$ integrin receptor in cell membranes via their RGD (Arg-Gly-Asn) sequence in the C-terminal domain, suggesting the possibility of an IGF-independent mechanism via these receptors (Jones *et al*, 1993a; Rauschnabel *et al*, 1999 abstract from Brighton meeting). Most of the IGFBPs have cell surface glycosaminoglycan, such as heparin and heparan sulphate, and extracellular matrix component binding properties (Hodgekinson *et al*, 1994). IGFBP-3 and –5 have the greatest potential to bind extracellular matrix via a stretch of basic amino acid in their C-terminal domain, and there is increasing evidence that IGFBP-3 and –5 have IGF-independent action through interaction of their heparin binding motifs and cell surface components (discussed later).

Adherence to cell surfaces, ECM proteoglycans, and glycosaminoglycans (GAGs) by various IGFBPs also has the potential to effect their affinity for binding IGFs. The ECM may serve

to create a favourable microenvironment for cellular proliferation by stabilising or increasing local concentrations of peptide growth factors, by protecting such peptides from proteolysis or even regulating the interaction of growth factors with target receptors. Jones *et al* (1993b) found that adding an increasing concentration of salt resulted in decreased binding of IGFBP-5 to fibroblast ECM. This clearly demonstrated that the binding of GAGs and/or proteoglycans to IGFBPs is due to charge dependent ionic bonds and is not a hydrophobic event. Therefore, charged amino acids in the IGFBP-5 protein are important for ECM binding.

Consensus sequences for GAG binding have been formulated from known heparin binding proteins and used to identify potential heparin-binding regions in a range of proteins (Cardin and Weintraub, 1989). They identified two types of heparin binding sequences: XBBXBX (S: short type) and XBBBXXBX (L: long type), where B represents any basic amino acid (mainly Lys or Arg) and where X is an unspecified amino acid. Based on this work, Hodgkinson *et al*, (1994) looked for potential GAG binding sites in all six IGFBPs through human and rat sequences (Figure I.5 and Table I.1). They found five of the IGFBPs have at least one putative consensus heparin binding site in their central domain and/or C-terminal domain (IGFBP-4 has none). Interestingly, IGFBP-5 contains three consensus heparin-binding sites, where the central domain has two overlapping S-type sequences and the C-terminal domain has one L-type sequence (Figure I.6). The GAG binding site in the C-terminal domain of IGFBP-3 (219-226) is conserved with that of IGFBP-5 (205-212). This region is part of a stretch of basic amino acids in the C-terminal domain of both IGFBP-3 and –5, suggesting a conserved biological function.

GAG and proteoglycan binding of IGFBP-3 and IGFBP-5 has been shown to play an important role in the ability of both of these IGFBPs to modify the cellular actions of IGF-I. However, the heparin binding capabilities of other IGFBPs has not as yet been reported, although the ability of IGFBP-2 to bind GAGs was shown to require that IGFBP-2 is already complexed with either IGF-I or –II (Arai *et al*, 1996b). Generally, IGFBP binding to GAGs/ECM modulates the affinity of the IGFBP for IGFs. For example, IGFBP-3 binding to cell surfaces GAGs was shown to result in a ten-fold reduction in affinity of the binding protein for IGF-I (McCusker *et al*, 1991). Also, IGFBP-5 binding to ECM caused up to a 17-fold reduction in affinity for IGF-I and

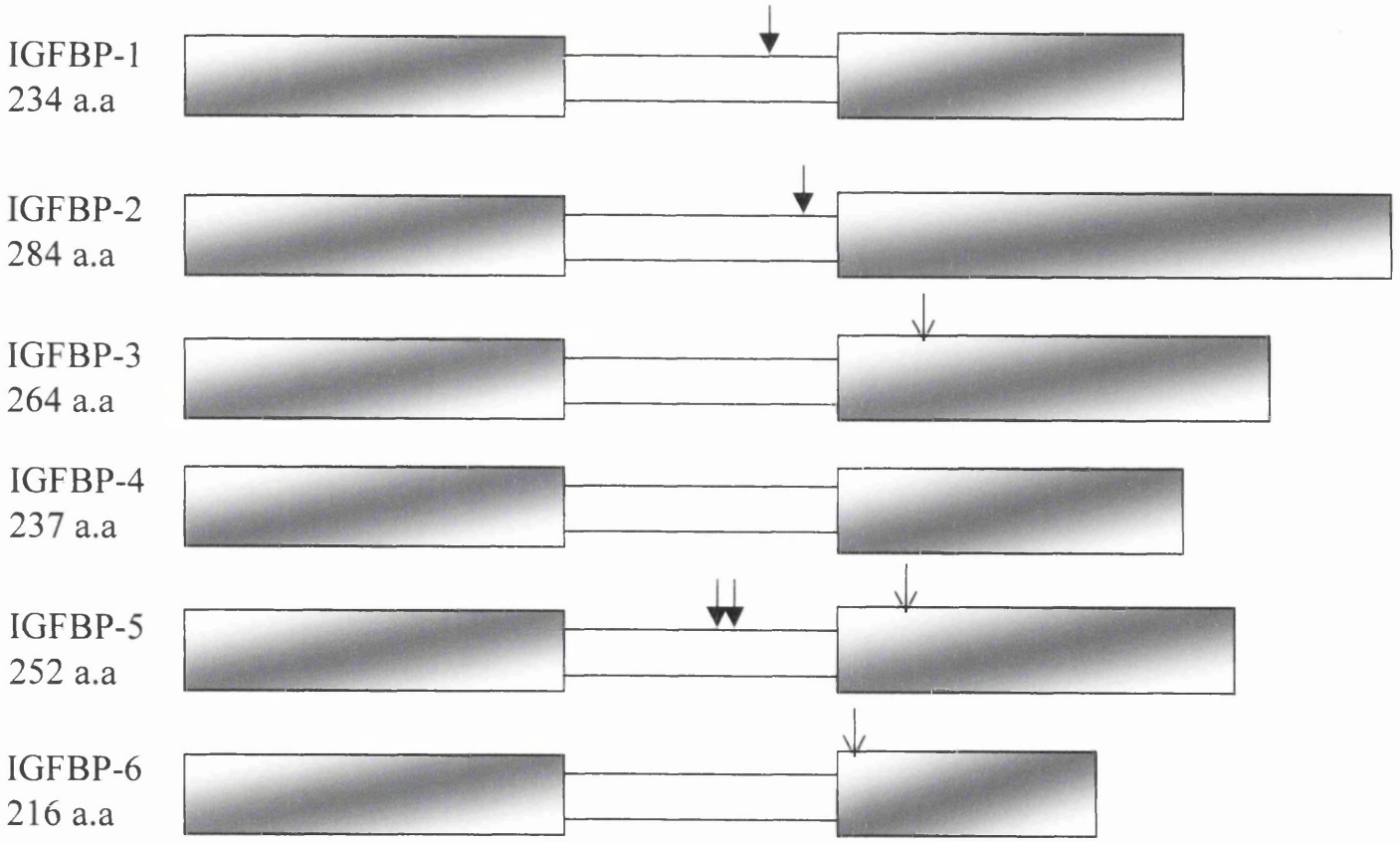
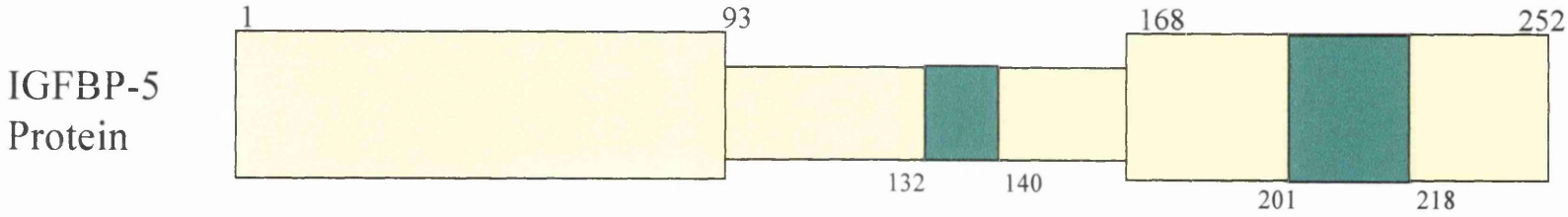


Figure I.5 Schematic representation of human IGFBP-1 to -6. The proteins are aligned by the first of the 18 homologous cysteine residues and the shaded area represent regions of high homology between the proteins. Locations of short and long GAG-binding consensus sequences are shown using solid and open arrows respectively.

Table I.1 GAG-binding consensus sequences of the IGFBP proteins
(Hodgekinson *et al*, 1994)

Protein	Sequence location (residue number)	Sequence type
Human		
IGFBP-1	144-149	S
IGFBP-2	179-184	S
IGFBP-3	219-226	L
IGFBP-4	-	-
IGFBP-5	132-137	S
	135-140	S
	205-212	L
IGFBP-6	172-179	L
Rat		
IGFBP-1	157-162	S
IGFBP-2	160-165	S
IGFBP-3	220-227	L
IGFBP-4	-	-
IGFBP-5	132-137	S
	135-140	S
	205-212	L
IGFBP-6	-	-

S and L refer to the short and long GAG-binding consensus sequences as originally described by Cardin & Weintrub (1989).
S = XBBXBX and L = XBBBXXBX where B represents Lys or Arg and X is undefined.



201 218

Arg-Lys-Gly-Phe-Tyr-Lys-Arg-Lys-Gln-Cys-Lys-Pro-Ser-Arg-Gly-Arg-Lys-Arg

L-type: **X B B B X X B X**

132 140

Val-Lys-Lys-Asp-Arg-Arg-Lys-Lys-Leu

S-type: **X B B X B X**

X B B X B X

Figure I.6 Central overlapping consensus heparin binding sites in IGFBP-5

was associated with stabilisation of the IGFBP-5/IGF complex such that it is resistant to proteolytic cleavage (Arai *et al*, 1994a).

The C-terminal domain 201-218 region of IGFBP-5 Association of IGFBP-5 with ECM results in an alteration in cell responsiveness to IGFs. The major binding site for ECM components (GAGs and proteoglycans) in the native IGFBP-5 protein is a stretch of basic amino acids (Arg^{201} to Arg^{218}) in the C-terminal domain (Figure I.6). Substitution of four basic amino acids within this region resulted in a major decrease in binding ECM components (Parker *et al*, 1998; Song *et al*, 2000).

Furthermore, the binding of IGFBP-5 with GAGs was shown to cause an 8-to 17-fold decrease in the affinity of IGFBP-5 for IGF-I (Jones *et al*, 1993b; Arai *et al*, 1994a; Parker *et al*, 1998). Arai *et al.* (1994a) suggested that GAGs did not directly compete with IGF-I for binding to IGFBP-5, but that GAG binding resulted in a conformational change in the IGFBP-5 protein structure, and that it was this that lowered its affinity for IGF-I. They also concluded that GAGs (and by inference the ECM) can lower IGF-I/IGFBP-5 interaction and this promotes IGF-I/receptor interaction. This may be crucial in obtaining the correct balance between a pool of ECM-bound IGFs that are protected from proteolysis and soluble IGFs able to interact with the cell surface receptor and bring about IGF effects.

More recently, Andress (1995) tested whether GAGs were also involved in the association of IGFBP-5 with the cell membrane. Both IGFBP-5 and its C-terminal-truncated variant $^{1\text{-}169}$ IGFBP-5 were shown to bind to osteoblast-like cell membranes and subsequently to become internalised. This binding was inhibited by a high salt concentration and could be competed with heparin> heparan sulphate> dermatan sulphate in decreasing order for both proteins (although higher salt and heparin concentration were required to displace $^{1\text{-}169}$ IGFBP-5 compared to the wild type protein). However, the conclusion that these results derive from a direct competition between the above molecules and cell membrane GAGs was called into question by the inability of heparinase or sodium chlorate treatment of the cell monolayers to affect IGFBP-5 binding to the cell membrane. The same group then went on to demonstrate that by cross-linking IGFBP-5 or $^{1\text{-}169}$ IGFBP-5 to osteoblast monolayers, it was possible to detect labelled proteins in a complex with a

420kDa Triton-extractable membrane protein. This protein was then purified from a cellular extract on an IGFBP-5 affinity chromatography column. The affinity labelling was greater for IGFBP-5 than for its truncated variant and the formation of the complex was not affected by heparinase or chondroitinase pretreatment of the cell monolayer. In contrast, IGFBP-5 and $^{1\text{-}169}$IGFBP-5 failed to cross-link to any protein from an ECM preparation. The authors concluded that, while IGFBP-5 association with the ECM might involve GAG interactions, the binding to osteoblast cell surfaces was mediated by a 420kDa protein, which could serve as an IGFBP-5 specific receptor. More recently, the functions of this putative receptor were further investigated (Andress, 1998). Treatment with IGFBP-5, or with the 201-218 peptide derived from IGFBP-5, was shown to cause increased serine phosphorylation of the 420kDa protein, which also appeared to display some kinase activity and be able to induce serine phosphorylation of casein *in vitro*.

It is well established that IGFBP-5 is the major bone IGFBP, and IGFBP-5 can bind to bone matrix, including the inorganic ECM component hydroxyapatite (HA). Campbell and Andress (1997b) showed that a synthetic IGFBP-5 (201-218) peptide was able to increase the binding of endogenous IGFBP-5 to HA as well as the binding of IGF-I to HA-bound IGFBP-5, and that this action was specific for the heparin binding domain. These results suggested that the 201-218 region of IGFBP-5 not only enhances binding of IGFBP-5 to HA, but that it also binds directly to IGFBP-5 in a certain way as to increase IGF-I binding to IGFBP-5. These data may indicate that IGFBP-5 is able to bind to itself, and suggests the possibility of dimer formation (Campbell and Andress, 1997b).

Domain switching to create chimeric IGFBP proteins has previously been adopted by other groups in order to ascertain which domains of IGFBP-3 and IGFBP-5 are involved in binding to a liver derived glycoprotein known as the ALS (Hashimoto *et al.* 1997, Twigg and Baxter, 1998). Using this approach, it was demonstrated that the stretch of basic amino acids that is common to the C-terminal domains of IGFBP-3 and IGFBP-5 is necessary for ternary complex formation with ALS and IGFs. In addition, it was shown that at high concentrations, the GAGs heparin and heparan sulphate inhibited ALS binding to IGFBP-5 when it was bound to IGF, suggesting overlap of heparin and ALS binding sites in the C-terminal domain (Twigg *et al.* 1998).

In addition to the functions of the 201-218 region outlined above, a nuclear localisation sequence (NLS) was also identified within this region of IGFBP-5 (Schedlich *et al.*, 1998; Schedlich *et al*, 2000), and an alternative route for IGFBP interaction with cells emerged (described in chapter I.4.4). Finally, the 201-218 region has been shown to be involved in the interaction between IGFBP-5 and plasminogen activator inhibitor 1 (PAI-1), part of the plasminogen activation pathway (Campbell and Andress, 1997a; Campbell *et al*, 1998), and αS2-casein (Flint *et al*, 2000).

The Central domain heparin binding region of IGFBP-5 Although a heparin binding consensus sequence has been identified in the central domain of IGFBP-5, its biological significance has not been explored fully. Parker *et al* (1996) synthesized central domain heparin binding peptides (Ala^{131}-Thr^{141}) and demonstrated that these could compete with C-terminal heparin binding peptides (Arg^{201}-Arg^{218}) for binding the ECM. However, they also noted that the central domain peptides were much less potent than the C-terminal domain peptides for binding ECM (Parker *et al*, 1996). Nevertheless, there is increasing evidence that the central domain heparin binding region of IGFBP-5 is activated upon removal of the C-terminal domain (Twigg *et al*, 2000; Song *et al*, 2001). Twigg *et al* (2000) generated a C-terminally truncated IGFBP-5, which is a mimic of a naturally occurring proteolytic fragment, and a domain switched chimeric mutant of IGFBP-5 and –6, termed 6-5-6. In both cases, mutant IGFBP-5 proteins formed ternary complexes with IGFs and ALS, suggesting that the central domain of IGFBP-5 contained the ALS binding activity. We have also shown that the central domain heparin binding region was activated after removal of C-terminal domain of IGFBP-5 (Song *et al*, 2001. This study will be discussed in more detail in chapter V). Furthermore, this suggests that proteolytic fragments of IGFBP-5 may retain significant ECM binding properties, and as a result may have differential effects to the native protein.

I.3.5 Post-translational modification

There is very little data on the effects of post-translational modification of IGFBPs on IGF binding. There is some evidence indicating that neither glycosylation nor phosphorylation appears to have a significant influence on the IGF binding affinities of IGFBPs (Hoeck and Mullu, 1994;

Coverley and Baxter, 1995). So far, post-translational modifications of the IGFBPs have been found in the central-domain, but not in the N- or C- terminal domains (Hwa *et al*, 1998).

Glycosylation of IGFBPs has been reported so far in IGFBP-3, -4, -5 and -6. There is no clear evidence to date that IGFBP-1 and -2 are glycosylated (Table I.2). Both N- and O-linked glycosylation has been reported; IGFBP-3 and -4 are N-glycosylated, whereas, IGFBP-5 and -6 are O-glycosylated. N-glycosylation appears only on an asparagine that is part of the consensus sequence Asn-X-Ser/Thr, where X is any amino acid except proline. Three N-glycosylation sites in the mature IGFBP-3 protein, Ans^{89}, Asn^{109} and Asn^{172} have been characterized (Zapf *et al*, 1988), and one, Asn104, in IGFBP-4 (Ceda *et al*, 1991). In contrast to the N-glycosylation sites, there are no consensus sequences for predicting O-glycosylation. Although, it has been reported that IGFBP-5 (Conover and Kiefer, 1993) and IGFBP-6 (Bach *et al*, 1992; Neumann *et al*, 1998) are both O-glycosylated, glycosylation sites in IGFBP-6 were determined to be within the central domain at 5 amino acids, Thr^{126}, Ser^{144}, Thr^{145}, Thr^{146}, and Ser^{152} (Neumann *et al*, 1998). The ability to bind IGFs with high affinity appears not be influenced by N- or O-glycosylation, although there may be effects on other functions of IGFBPs, such as resistance to proteolysis (Neumann *et al*, 1998).

IGFBP-1, -3, and –5 are also post-translationally modified by ***phosphorylation*** (Coverley and Baxter, 1997). Phosphorylation in all three IGFBPs is predominantly at serine residues found in the central domain (Table I.2). Phosphorylation of proteins is an important and critical post-translational modification mechanism that is used by cells to stringently regulate the activities of numerous intracellular proteins, including proteins involved in the signal transduction pathways, in the cell cycle, and in gene expression. The purpose of phosphorylating secreted proteins like IGFBPs is unclear, but there is evidence that, at least for human IGFBP-1, phosphorylation enhances the affinity of this binding protein for IGFs 5-fold (Jones *et al*, 1991; Westwood *et al*, 1997). The phosphorylation of human IGFBP-1 occurs at three serine residues: Ser101 and Ser109 in the central domain, and Ser169 in the C-terminal domain. The phosphorylation of human IGFBP-3 was found at Ser111 and Ser113 (Hoeck and Mukku, 1994). Both of these serines are also in close proximity to one of the N-glycosylation sites, Asn109. This work also demonstrated that phosphorylation of human IGFBP-3 did not appear to affect IGF binding (Hoeck and Mukku, 1994), although interestingly the phosphorylation of IGFBP-3 can be up-regulated by IGFs through

Table 1.2 (based on Jones and Clemmons, 1995a; LeRoith, 1996; Hodgkinson *et al.*, 1994 and Swiss-Prot sequence data base)

Characteristics	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6
N residues						
Human	234	289	264	237	252	249
Rat	247	270	265	233	252	252
Mr theoretical						
Human	27903	35137	31660	27934	28553	30570
Rat	29628	32883	31680	27745	28428	30298
SDS-page (non reducing) ≈	30	32	39-41	24-28	30	30
Phosphorylation	+	-	+	-	+	-
Glycosylation	-	-	N	N	O	O
RGD sequence	+	+	-	-	-	-
Heparin-bind (S= short; L=long)	S	S	S + L	nd	S+S+L	Nd
Other basic aa seq.			215-232		201-218	
Heparin binding	+/-	+	++	+	++	Nd
Cell surf. Associat.	+	+	+	-	+	-
Proteolysis	-	+	+	+	+	Nd
IGF-I-IGF-II preference	≈	≥IGF-I	≥IGF-II	≈	≥IGF-I	>>IGF-II
Chromosomal localiz. (human)	7p	2q	7p	17q	2q	12
HOX gene association	HOXA	HOXD	HOXA	HOXB	HOXD	HOXC

Nd = not determined

a mechanism involving IGF-I-R interaction (Coverley and Baxter, 1995). The importance of the phosphorylation of IGFBP-3, and the significance of IGF regulation of phosphorylation, is unclear but it may affect IGFBP-3 interactions with the ALS or with the cell surface (Coverley and Baxter, 1995).

The data for the phosphorylation of IGFBP-5 are very limited, although, like all the IGFBPs, there are several potential phosphorylation sites at serine and threonine residues in the central domain (Coverley and Baxter, 1995). The biological significance of IGFBP-5 phosphorylation has not been elucidated.

I.3.6 Proteolysis of IGFBPs

Many studies have shown that proteolysis is an important mechanism for generating IGFBP fragments with a lower affinity for IGFs. Cleavage of IGFBPs results in release of free IGFs, which are then able to activate the IGF receptors (FigureI.1). Prostate specific antigen (PSA) in seminal plasma was the first IGFBP protease to be biochemically identified (Cohen *et al*, 1992). To date, proteolysis of IGFBP-2 to –6 has been described in multiple clinical states and cellular systems. Categories of IGFBP protease include kallikrein-like serine proteases (Cohen *et al*, 1992; Rajah *et al*, 1996), cathepsins (Conover *et al*, 1994; Conover *et al*, 1995b; Nunn *et al*, 1997; Claussen *et al*, 1997), and matrix metalloproteinases (MMPs) (Fowlkes *et al*, 1994a-b; Rajah *et al*, 1996). Kallikrein-like serine proteases include PSA, gamma-nerve growth factor and plasmin (Angelloz-Nicoud *et al*, 1996). PSA is present in high concentrations in patients with disseminated prostate cancer (Cohen *et al*, 1992), that is able to degrade IGFBP-3 and –5, but not IGFBP-1, -2, -4 or –6 (Collett-Solberg and Cohen, 1996). In addition, a serine protease specific to IGFBP-4 has been observed in smooth muscle and neuronal cell lines (Rees *et al*, 1998), and thrombin, another serine protease, cleaves IGFBP-5 at physiologically relevant concentrations (Zheng *et al*, 1998a). Cathepsins are proteases which are activated under acidic conditions, and this may be relevant to certain physiological and pathological processes (Conover and De Leon, 1994). Under such conditions, a high release of hydrogen ions may provide an acidic environment for extracellular cathepsin action, resulting in the cleavage of IGFBPs, which, in turn, may impact on cell growth

rates through interaction with the IGF axis (Bereket *et al*, 1996). *In vitro* degradation experiments of IGFBPs by cathepsin D showed that IGFBP-1 to –5 were cleaved by cathepsin D in a concentration dependent manner to fragments of a defined size, (IGFBP-6 was not degraded) (Claussen *et al*, 1997). MMPs comprise a family of peptide hydrolases responsible for the degradation of ECM components such as collagen and proteoglycans in processes involving tissue remodeling (Fowlkes *et al*, 1994a). MMPs have been identified in prostatic fluid and cells (Nunn *et al*, 1997). The fact that certain IGFBPs have been shown to be degraded by MMPs may imply a role for the binding proteins in these tissue remodeling events.

Proteolysis of IGFBP-5 has been the subject of study in many laboratories. IGFBP-5 proteases belonging to the families of matrix metalloproteases and serine proteases have been described in fibroblast, osteoblast and chondrocyte culture (Kanzaki *et al*, 1994; Nam *et al*, 1994; Fowlkes *et al*, 1994a; Thraikill *et al*, 1995a; Matsumoto *et al*, 1996). Nam *et al.* (1994) identified an IGFBP-5 specific calcium-dependent serine protease in the culture medium from human fibroblasts, which cleaves intact IGFBP-5 to 17-22 kDa fragments. The proteolytic specificity for IGFBP-5 was confirmed by experiments in which IGFBP-1, -2, -3 and –4 were shown to remain intact after incubation with purified protease. The authors went on to show that the protease has properties similar to those of members of the kallikrein family. It is calcium and not zinc dependent, and its protease inhibitor activity profile shows that aprotinin is one of the most effective inhibitors. Most importantly, its activity is inhibited by a peptide containing the α_1-antichymotrypsin cleavage site (Yoon *et al*, 1987). α_1-Antichymotrypsin is a member of the serpin family of protease inhibitors, and it inhibits members of the kallikrein family (Huber and Carrel, 1989). However, the IGFBP-5 protease does not appear to be a known kallikrein, because plasma kallikrein had minimal activity, and cleavage by this protease produced different fragment sizes. Likewise, IGFBP-5 protease activity was not inhibited by soybean trypsin inhibitor, a known kallikrein inhibitor. Furthermore, PSA cleaves IGFBP-3 (Watt *et al*, 1986), whereas the IGFBP-5 protease does not cleave this substrate. These finding suggest that the protease may be similar to human kallikreins, but two members of this family have been excluded as candidates.

Subsequently, Imai *et al.* (1997) identified the same protease in the culture medium from porcine smooth muscle cells (pSMC) and used site-directed mutagenesis to identify the primary

proteolytic cleavage site at Arg^{138}-Arg^{139}, (which interestingly lies within the consensus heparin binding site in the central domain of IGFBP-5; previously discussed in chapter 1.3.4). By mutagenesis of these residues, (Arg^{138}-Arg^{139} → Asn^{138}-Asn^{139}), the authors were able to create a protease resistant mutant of IGFBP-5, which remained intact even after 24hr of incubation with the pSMC protease. Furthermore, the mutant IGFBP-5 was shown to inhibit several IGF-I actions when added to pSMC culture medium, including decreasing IGF-I stimulated cellular DNA synthesis by 84%, protein synthesis by 77%, inhibition of IGF-I stimulated migration of pSMC by 77%, and inhibition of IGF-I stimulated phosphorylation of Insulin receptor substrate-1 (IRS-1). In contrast, the same amount of native IGFBP-5 did not have the same inhibitory effects on IGF-I actions. Therefore, these findings suggest that the proteolysis of IGFBP-5 is essential in order to potentiate several IGF-I actions, probably via the release of IGF-I to its receptor.

Interestingly, Nam *et al* (1994) also showed that the proteolysis of IGFBP-5 by its specific protease was inhibited by co-incubation with either heparin or a synthetic C-terminal domain basic amino acid region (201-218) peptide, and the authors concluded that heparin and the peptide may both bind directly to the protease through a charge based interaction. Initially, this conclusion appears surprising as heparin and the basic amino rich peptide have opposite charges (negative and positive respectively), which promotes their own interaction. In order for this to occur, it would be necessary for the protease to contain both positively and negatively charged regions to enable it to bind both of these molecules. We would consider the 201-218 peptide to be a likely binding partner for the protease, because the primary proteolytic cleavage site lies within a similar stretch of basic amino acids (the central heparin binding consensus site). Therefore, it is conceivable that the 201-218 peptide binds the protease directly and has an inhibitory effect. However, it is possible that heparin interferes with IGFBP-5 proteolytic activity by directly binding to either the IGFBP-5 or the protease. In support of the former, it has been shown that IGFBP-5 bound to ECM components through the 201-218 region is protected from subsequent proteolysis (Parker *et al*, 1998). Therefore, heparin binding to the 201-218 region of IGFBP-5 may have a conformational effect on the protein structure that prevents proteolysis in the central domain.

In addition to the IGFBP-5 specific protease, it has also been shown that the binding protein is cleaved by physiological relevant concentrations of thrombin (Zheng *et al*, 1998a). In

addition, the authors were able to identify three thrombin cleavage sites in the IGFBP-5 protein (Lys120-His121, Arg156-Ile157, and Arg192-Ala193), two of which are C-terminal to the central heparin binding consensus sequence. Finally, the authors demonstrated that thrombin cleavage of IGFBP-5 was partially inhibited by IGF-I binding and they suggested that generation of active thrombin in vessel walls may be a physiologically relevant mechanism for controlling IGF-I bioactivity. Another group identified IGFBP-5 proteases of molecular weights 52-72 kDa in the culture medium of MC3T3-E1 osteoblasts, and subsequently were able to show that these proteases were MMPs (Thrailkill *et al*, 1995a). The cleavage sites for the MMPs in the IGFBP-5 protein have yet to be identified. Finally, screening of circulating 12 to 25kDa human IGFBP-5 fragments, which have very low affinity for IGFs as detected by ligand blots, in human hemofiltrate revealed further proteolysis sites in the IGFBP-5 sequence at Ser143, Lys144, and Arg188 (Standker *et al*, 1998). Interestingly, only the 13.5kDa fragment was derived from the C-terminal domain, whereas most of the major fragments (Mr >20kDa) contained the central domain. These findings suggest that most of the primary cleavage sites are located in the central domain.

I.4 IGFBP Functions

IGFBPs have been shown to either inhibit or enhance IGF actions in different circumstances (reviewed in Hwa *et al*, 1999). The functions of IGFBPs and IGF actions have been studied in detail, but most of these studies have been conducted *in vitro* using cultured cells. Recently, *in vivo* studies of IGFBP action have also been carried out. These *in vitro* and *in vivo* studies have allowed a more comprehensive understanding of the role of IGFBPs in controlling IGF action. Although this section will discuss the individual actions of each protein, since they have usually been studied individually, it is important to remember that most cell types synthesize multiple IGFBP types as well as IGFBP proteases. Another fact that is often overlooked is that most *in vitro* experiments are conducted by removing the conditioned culture medium from the cell cultures at the time of initiation of the experiment, thereby reducing the soluble binding protein levels in the medium to zero before adding back IGFs. This is a nonphysiological situation since IGFBPs are present in all interstitial fluids *in vivo*. There is also increasing evidence for IGFBPs having IGF-independent activities mediated through their own receptors.

I.4.1 *In vivo* functions

In plasma there are three general functions of IGFBPs; 1) The IGFBPs transport IGFs to their receptor sites in the body, 2) The binding of IGFBPs prolongs IGF half-life by as much as 30min, or to 16hr when bound to IGFBP-3 in the 150kDa complex, compared to 10min or less for free IGF alone (Jones and Clemmons, 1995), 3) Complexing with IGFBPs inhibits the insulin-like activity of IGFs and prevents hypoglycemia that would otherwise lead to death (Chan and Spencer, 1997). The IGFs are carried in the circulation predominantly in the ~150kDa complex with IGFBP-3 and the ALS, which does not bind IGFs directly. This complex sequesters more than 90% of the IGFs in the circulation (Lee and Rechler, 1996). Most of the rest of the IGFs are bound to other IGFBPs and approximately 0.4% remains in the free form (Clemmons, 1997). ALS is a glycoprotein with an apparent molecular weight of 84-86 kDa and a characteristic leucine-rich domain which is believed to facilitate protein-protein interaction (Leong *et al.*, 1992). Liver is the main source of plasma IGF, IGFBP-3 and ALS. The proteins are released into the circulation where IGF initially combines with IGFBP-3 in a dimer complex and then subsequently binds to the ALS subunit to form a stable 150 kDa ternary complex (Jones and Clemmons, 1995). Recently, it was reported that a plasminogen and IGFBP-3 complex is also able to bind IGF-I with high affinity in normal plasma (Campbell, 1998). The theory that all IGFBP-3 present in the plasma is bound to ALS and IGF was based on gel filtration studies that simply showed that plasma IGFBP-3 is engaged in a 150 kDa complex. However, as plasminogen (97-92 kDa) has a similar molecular weight to ALS, it is possible that part of the 150 kDa is actually formed by IGFBP-3, IGF and plasminogen. Furthermore, an IGFBP-3-ALS-plasminogen trimeric complex could not form as both ALS and plasminogen interact with the 215-232 heparin binding sequence in the C-terminus of IGFBP-3, and therefore, their binding to IGFBP-3 is mutually exclusive.

It is believed that IGFBPs can also play a role in IGF transcapillary transport. Low molecular weight IGF-IGFBP complexes have been shown to cross capillary barriers (Jones and Clemmons, 1995) and a specific role for endothelium associated IGFBP-3 fragments has been proposed (Booth *et al*, 1996). IGFBP-1 has been identified in peritoneal fluid, the composition of which is believed to be similar to interstitial fluid. It is not known if the passage of IGFBP-1 to the

extravascular compartment occurs by passive leakage from the blood or if it involves an active/regulated transport (Lee *et al.*, 1997).

The deletion of IGF-I and -II mouse genes by homologous recombination produced animals of reduced in size (DeChiara *et al.*, 1990; Powell-Braxton *et al.*, 1993) indicating the involvement of the two peptides as cell survival factors in embryonic development. A role for IGFBPs in development was also postulated, acting either through the modulation of IGF action or in an IGF-independent fashion. IGFBP mRNA expression in rat and mouse embryos has been determined by *in situ* hybridization techniques and specific temporal-spatial expression patterns were observed (Baker *et al*, 1993; Powell-Braxton *et al*, 1993; Cerro *et al*, 1993; Green *et al*, 1994; Schuller *et al*, 1993; Streck *et al*, 1992; Wood *et al*, 1992). In particular, IGFBP-2 and-5 expression appears to be localized in the same or adjacent tissues (Green *et al*, 1994; Schuller *et al*, 1993) suggesting a complementary role for these two binding proteins in specific tissues during development (Allan *et al*, 2000). However, targeted disruption of the IGFBP-2 gene in mice did not produce any apparent phenotypical alteration (Wood *et al*, 1993). This result, while possibly suggesting functional redundancy between the 6 IGFPBs, does not exclude a specific role for each IGF-binding protein in the development of normal embryos.

1.4.2 Inhibition of IGFs action

The diverse activities of IGF-I and IGF-II in stimulating mitogenesis, increasing substrate uptake and metabolic activity, inhibiting apoptosis, and modulating a variety of specific functions in highly differentiated cell types are mediated through binding and activation of the type I IGF receptor. Although there are notable exceptions, the interaction of an IGFBP with IGF-I or IGF-II generally blocks receptor activation.

IGFBP-1. As reviewed in Clemmons (1997), many studies have demonstrated the inhibitory effects of IGFBP-1 on IGFs. Addition of molar excess of IGFBP-1 with IGF-I or IGF-II (at least 4:1) results in inhibition of IGF action, and excess IGFBP-1 has been shown to inhibit protein synthesis in muscle cells (McCusker *et al*, 1991; reviewed by Clemmons, 1997). One experiment has shown that IGFBP-1 blocks IGF-I interaction with receptors on human endometrial

membranes, suggesting that this is the principal mechanism by which inhibition occurs (Rutanen *et al*, 1988). Prolonged incubation times of 24-36h with highly phoshorylated forms of IGFBP-1 have been shown to inhibit DNA synthesis in FRTL5 cells and chick embryo fibroblasts (Frauman *et al*, 1989). Also of interest was that while phosphorylated IGFBP-1 decreased IGF-I-stimulated DNA synthesis in human fibroblasts, dephosphorylated IGFBP-1 actually potentiated IGF action (Busby *et al.*, 1988; Jones and Clemmons, 1995). These differential effects may be explained by the fact that phosphorylation of IGFBP-1 increases its affinity for binding IGFs 6-fold, and this would also increase the difference in IGF-affinity between IGFBP-1 and the type-1 IGF receptor (Jones and Clemmons, 1995).

Recently, Gustafsson *et al.* (1999) demonstrated how IGFBP-1, -2 and –4 modulate IGF-I-induced DNA and protein synthesis in cultured rat vascular smooth muscle cells. DNA and protein synthesis were measured as incorporation of ^{3}H-thymidine and ^{3}H-leucine into DNA and protein respectively. IGFBP-1 and –4 acted in an inhibitory manner on IGF-I-induced DNA synthesis. While IGFBP-2 also inhibited the growth response to IGF-I, this effect was only obtained if the IGFBP-2 and IGF-I were pre-incubated together for 2hr prior to their addition to the cells. IGF-I-induced protein synthesis was also inhibited by all three IGFBPs. Similar results were reported by another group who showed that IGFBP-1 inhibits IGF-II stimulated DNA synthesis and the extent of glycogenesis in cultured rat hepatocytes (Menuelle *et al*, 1999).

Some *in vivo* and clinical studies have also shown inhibitory effects of IGFBP-1. Lewitt *et al.* (1991) injected a very large concentration of IGFBP-1 into rats, which resulted in at least a 10:1 excess of IGFBP-1 to free IGF-I. This excess of IGFBP-1 resulted in a transient increase of blood glucose concentration, which suggests that molar excess of IGFBP-1 inhibits IGF-I mediated glucose uptake. In addition, increased IGFBP-1 levels were found in a patient who had displayed severe growth retardation since birth (Camacho-Hubner *et al*, 1999). It was found that in addition to inhibiting the biological activity of the IGFs, the increased IGFBP-1 levels were also associated with inhibition of circulating 150kDa ternary complex formation, which in turn would accelerate the rate of clearance of IGFs. Related to this, it has also been shown that increased IGFBP-1 levels inhibits the feedback action on GH leading to increased levels of this hormone (Barreca *et al*, 1998). This could lead to GH insensitivity syndrome, which also inhibits body growth.

In several transgenic studies, overexpression of IGFBP-1 in the brain caused a retardation in brain growth (Dai *et al*, 1994). Interestingly, although the IGFBP-1 level in serum was ten times higher than in the wild type controls, the transgenic mice showed no growth retardation, suggesting that the molar excess of IGFBP-1 to IGF-I in the brain was relatively greater than in the serum. Lee *et al.* (1999) showed that IGF-I transgenic mice had enhanced growth even in undernourished conditions, whereas undernourished IGFBP-1 transgenic mice exhibited less growth than undernourished control mice. These *in vivo* experiments also illustrate that, as with in-vitro conditions, high levels of IGFBP-1 relative to IGF-I can result in growth attenuation.

IGFBP-2. As reviewed in Clemmons (1997), IGFBP-2 is secreted by many cell lines and appears to be involved in cell proliferation. In most of the cell culture systems studied, IGFBP-2 accumulates in the conditioned medium where it binds to IGFs with high affinity and potentially inhibits IGF action by preventing the interaction of the growth factors with the IGF-I receptor. In rat calvaria cells IGFBP-2 reduced both basal and IGF-I-stimulated ^{3}[H]-thymidine uptake and collagen synthesis (Feyen *et al*, 1991). In porcine muscle satellite cells, multiple passaging culture caused reduced cell division and myotube formation accompanied by an increased secretion of IGFBP-2, which in turn may have reduced endogenous IGF-I activity (Fligger *et al*, 1998).

The addition of plasminogen to neuroblastoma cell cultures caused proteolysis of IGFBP-2 which resulted in a 4-5 fold reduction in the affinity of the binding protein for IGF-II. In these cells, IGFBP-2 is the major regulator of IGF-II action as it is more abundant than IGFBP-4, which is also expressed and it has higher affinity for IGF-II. Plasminogen also induced a mitogenic effect in neuroblastoma cells, which was inhibited by blocking with an IGF-II specific antibody. This led to the conclusion that the plasminogen mitogenic effect derived from the increased bioavailability of IGF-II following the degradation of IGFBP-2 (Menouny *et al*, 1997).

Analogous experiments were undertaken in kidney fibroblasts stably transfected with an IGFBP-2 expression vector. The growth rate of cells transfected with IGFBP-2 was reduced compared to the control cells and the inhibition was reversed by IGF-I and II and even more potently by an IGF-I analogue, des[1-3]IGF-I, which has reduced affinity for IGFBPs. The anti-proliferating effect of IGFBP-2 was confirmed when the medium conditioned by IGFBP-2-

transfected kidney fibroblasts was applied to choriocarcinoma cells, causing a reduction of cell proliferation (Hoflich *et al*, 1998)

A recently published study analyzed the correlation between the level of expression of IGFBP-2 and growth rate in C6 rat glioma cells transfected with an IGFBP-2 expression vector (Bradshaw *et al*, 1999). That IGFBP-2 might play a role in the regulation of cell proliferation was suggested by experiments in which primary cultures of glioma cells was transfected with IGFBP-2. Clones of cells with high levels of IGFBP-2 expression had decreased proliferation rates, whereas high rates of proliferation were associated with low levels of IGFBP-2 expression. Moreover in a previous report the authors showed that the addition of IGFBP-2 to primary astroglial cells caused an inhibition of IGF stimulated ^{3}[H]-thymidine uptake (Bradshaw and Han, 1993). Therefore, it was expected that the higher the expression of IGFBP-2 in C6 transfected clones, the lower was the rate of cell proliferation. Surprisingly the growth rate of cloned cells that expressed the highest level of IGFBP-2 did not differ from control cells, while an inhibition of cell proliferation became evident in clones expressing lower levels of the binding protein. Other parameters were also analyzed and interesting observations were recorded. First, the clones that expressed IGFBP-2 at the highest level displayed a concomitant upregulation of the endogenous IGF-I mRNA and it is possible that this cell compensation mechanism limited the effect of IGFBP-2 on cell proliferation. Second, the transfection of glioma cells with IGFBP-2 vector affected the expression pattern of other endogenously produced IGFBPs. For instance, in the clones with lower growth rate and moderate IGFBP-2 expression levels the appearance of a new 29kDa binding protein (possibly IGFBP-5) was noted both in the conditioned medium and associated to the cell membranes. Although transfected clones moderately expressing IGFBP-2 showed a decrease in cell proliferation it is possible that the effect was due to the induction of IGFBP-5 expression. It was concluded that factors other than the equilibrium between IGF-I and IGFBP-2 were involved in growth regulation (Bradshaw *et al*, 1999).

IGFBP-3. As reviewed in Clemmons (1997), addition of excess purified IGFBP-3 with IGF-I inhibits the insulin-like actions of IGF-I in Balb/c 3T3 cells. In cultured fibroblasts, excess IGFBP-3 inhibits the DNA synthesis response to IGF-I with maximum inhibition noted at a 4:1 molar ratio of IGFBP-3 to IGF-I (Blat *et al*, 1989). IGFBP-3 has been shown in chick fibroblasts to

inhibit IGF-I stimulated DNA synthesis (Villaudy *et al*, 1991), and in rat granulosa cells steroidogenesis and cyclic AMP generation are inhibited by exogenous IGFBP-3 (Bicsak *et al*, 1990). In cultured rat or mouse osteoblasts, collagen synthesis has been shown to be inhibited by IGFBP-3 (Schmid *et al*, 1991), and it was proposed that the increased concentration of IGFBP-3 in the conditioned medium in mouse embryo fibroblasts could account for the observed density dependent inhibition of cell growth (Blat *et al*, 1994).

Exogenous IGFBP-3 in concentrations of 50ng/ml inhibited DNA synthesis induced by 1nM IGF-I in human vascular smooth muscle cells cultured from human renal arteries (Andersson *et al*, 1999). Moreover, the stimulatory effect of IGF-I through its receptor was shown to be inhibited significantly by addition of IGFBP-3 in human chondrocyte cells (Matsumoto, 2000). In a human glioblastoma cell line, the induction of p53 gene expression was shown to not only cause apoptosis, but also the stimulation of IGFBP-3 gene promoter and protein expression (Shen and Glazer, 1998). It had been shown previously that in this cell line the addition of recombinant IGFBP-3 caused apoptosis (possibly by inhibiting the cell survival action of the IGFs), but it remains to be demonstrated if the p53-induced apoptosis is mediated through the increase of endogenous IGFBP-3. Karas *et al.* (1997) showed that in endometrial cancer cells, membrane-associated IGFBP-3 inhibited IGF induced phosphorylation of IGF-I-R and IRS-1. Finally, TGF-β stimulates IGFBP-3 mRNA and peptide expression in several cell types, and TGF-β induced growth inhibition and apoptosis have been shown to be mediated through the induction of IGFBP-3 (Cohen, 2000).

IGFBP-4. To date, IGFBP-4 is the only IGFBP that has been shown to only inhibit IGFs. IGFBP-4 displays none of the properties associated with lowering of IGFBP affinity for IGFs, such as cell surface association or dephosphorylation. As reviewed in Clemmons (1997), however, inhibitory effects of IGFBP-4 are IGF-I-R dependent, including inhibition of IGF-I effects on DNA synthesis in cyclic AMP generation in osteoblasts (Mohan and Baylink, 1991), protein synthesis in B104 cells (Cheung *et al*, 1991), glycogen synthesis in osteosarcoma cells (Jones and Clemmons, 1995). Clinical observations have also reported that circulating levels of IGFBP-4 are increased in bone marrow with age, suggesting that IGFBP-4 has a negative effect on bone formation (Mohan *et al*, 1995).

Damon *et al* (1998) overexpressed IGFBP-4 in the M12 prostate epithelial cell line to determine the effects on tumor formation and apoptosis. IGF induced proliferation was reduced in the IGFBP-4 transfected cells compared with control cells. In addition, colony formation in soft agar was significantly inhibited in the IGFBP-4 transfected cells by up to 14 days after plating compared with the M12 controls, whereas, in the presence of des(1-3)IGF-I, which does not interact with IGFBPs but retains wild type potency at the IGF-I receptor, there was no significant difference between the control and IGFBP-4 transfectants in colony formation.

IGFBP-4 transgenic mice exhibited a significant reduction in wet weight of smooth muscle cell (SMC) rich tissues, including bladder, intestine, aorta, uterus, and stomach, with no change in total body or carcass weight (Wang *et al*, 1998). *In situ* hybridization showed that transgene expression was targeted exclusively to the muscular layers of the arteries, veins, bladder, stomach, intestine, and uterus. Overexpression of IGFBP-4 was associated with SMC hypoplasia, a reciprocal phenotype to that of transgenic mice overexpressing IGF-I under the control of the same promoter. Double transgenic mice derived from mating IGFBP-4 with IGF-1 transgenic animals show a modest decrease in wet weight at selected SMC tissues. These data suggest that IGFBP-4 is a functional antagonist of IGF-I action on SMC *in vivo*. In support of this, others have shown that IGFBP-4 can inhibit IGF-I induced DNA synthesis in human vascular smooth muscle cells (Andersson *et al*, 1999).

IGFBP-6. Due to its low affinity for IGF-I, any IGFBP-6 inhibitory effects are mainly restricted to IGF-II action. IGFBP-6 is an O-linked glycoprotein, and Marinaro, *et al* (2000) reported that O-glycosylation stimulates the inhibitory effects of IGFBP-6. The half-life of O-glycosylated IGFBP-6 was 2.3 fold greater than that of non-glycosylated IGFBP-6, however this O-glycosylation did not inhibit intravascular proteolysis of IGFBP-6, suggesting that O-glycosylation delays the clearance of IGFBP-6 from the circulation and therefore contribute to its role as an inhibitor of IGF-II actions. In the ovary, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) stimulate IGFBP-6 secretion which in turn inhibits IGF-I stimulated steroidogenesis, although less potently then IGFBP-2 or 4 (Jones and Clemmons, 1995). IGFBP-6 also inhibits IGF-II, but not IGF-I, induced differentiation of myoblasts (Bach *et al*, 1994). As described for other

IGFBPs, IGFBP-6 expression can be subjected to TGF-β regulation which has been shown to decrease IGFBP-6 mRNA in osteoblasts (Gabbitas and Canalis, 1997).

IGFBP-6 secretion in keratinocytes, myoblasts and neuroblastoma cells increases when cells undergo growth arrest (Chambery *et al*, 1998). These authors were also able to show that differentiation of neuroblastoma cells induced by retinoic acid caused a reduction in IGFBP-2 and -4 expression, but increased IGFBP-6 expression. They postulated that the function of IGFBP-6 in these cells might be to sequester endogenous IGF-II from interacting with the IGF-I-R. Overall, from *both in vitro* and *in vivo* studies, there is an indication that IGFBP-6 might play a growth inhibition role in a wide range of cell tumours (breast carcinoma, prostate carcinoma, endometrial carcinoma, neuroblastoma) (Sheikh *et al*, 1993; Bach and Rechler, 1996; Drivdahl *et al*, 1995; Gong *et al*, 1992; Babajko and Binoux, 1996).

IGFBP-5. As reviewed in Clemmons (1997), IGFBP-5 was shown to inhibit IGF-I and II-stimulated DNA and glycogen synthesis in human osteosarcoma cells (Kiefer *et al*, 1992), and decrease the IGF-I stimulated steroidogenesis in Leydig cells (Liu *et al*, 1993b). In addition, when IGFBP-5 was added in the presence of IGF-II, it inhibited both proliferation and differentiation in L6A1 muscle cells, whereas interestingly, in the presence of IGF-I, it stimulated all aspects of the myogenic response to the growth factor (Ewton *et al*, 1998).

In some of the experiments designed to investigate the biology of this binding protein it was noted that a correlation existed between the occurrence of apoptosis and the increased expression of IGFBP-5. Guennette and Tenniswood (1994) reported that IGFBP-5 expression increases in involuting prostate concomitant with apoptosis in this tissue. In addition, a correlation between IGFBP-5 expression and apoptosis was also noted in finasteride or castration-induced prostate involution (Thomas *et al*, 1998), in ovarian atretic follicles (Erickson *et al*., 1992), in rat involuting mammary gland (Tonner *et al*, 1995, 1997) and in the involuting thyroid (Phillips *et al*, 1994). Guennette and Tenniswood (1994) proposed a model to explain the specific role played by different IGFBPs during the involution of the prostate gland, and this is discussed below.

Pro-apoptotic model for IGFBP-5 action. In this model it was postulated that IGFBP-5 could exert a pro-apoptotic effect by sequestering the IGFs to the ECM and thereby preventing the interaction of the growth factors with IGF-I-R on the cell surface. In turn, this would inhibit the

cell survival signalling action of the IGFs and allow apoptosis to proceed. (Figure I.7). To exemplify this model, we will use the association of IGFBP-5 expression with apoptosis of mammary epithelial cells in the involuting mammary gland, as this is part of the ongoing research programme within our laboratory.

First, it has been established IGF-I can act as a survival factor for many different cell types including mammary epithelium (O'Conner, 1998). Several transgenic models have confirmed this survival effect of IGF-I, where overexpression of this growth factor within the mammary gland was shown to retard mammary gland involution after removal of the suckling young (Hadsell *et al*, 1996; Neuenschwander *et al*, 1996). Interestingly, IGFBP-5 expression in the lactating mammary gland has been shown to occur at very low level, comparable to that found in normal serum (Tonner *et al*, 1997). However, these authors demonstrated that there was a very large increase in both IGFBP-5 mRNA and protein expression within the involuting mammary gland, concomitant with apoptosis of mammary epithelial cells. Therefore, they hypothesized that one of the early events associated with mammary gland involution is the secretion of IGFBP-5, which in turn serves to inhibit IGF-I-mediated cell survival. In support of these findings, others have also shown that prolactin, which suppresses IGFBP-5 expression, can inhibit apoptosis (Travers *et al*, 1996), whereas teat sealing, which should increase IGFBP-5 expression, is associated with increased levels of apoptosis (Quarrie *et al*, 1996). However, Lund *et al* (1996) demonstrated that glucocorticoids, which do not have a demonstrated relationship with IGFBP-5 expression, only effect the later, second phase of involution occurring 3-4 days after litter removal, and this is consistent with the hypothesis that IGFBP-5 is involved in the initial stages of apoptosis.

A high affinity interaction has also been demonstrated between IGFBP-5 and the casein micelle fraction of milk (Tonner *et al*, 1997). Indeed, IGFBP-5 binds with high affinity to hydroxyapatite (a crystalline form of calcium phosphate) in bone (Mohan, 1993), and casein micelles also possess calcium phosphate nanoclusters (Holt, 1985). Therefore, as an alternative to IGFBP-5 sequestering IGFs to the ECM, it has been suggested that the binding protein may induce apoptosis of mammary epithelial cells by sequestering IGF-I to casein micelles, thereby inhibiting its survival function. However, the interaction of IGFBP-5 with the mammary epithelial cell surface cannot be ruled out, as has been proposed for endothelial cells (Booth *et al*, 1995), or the possibility

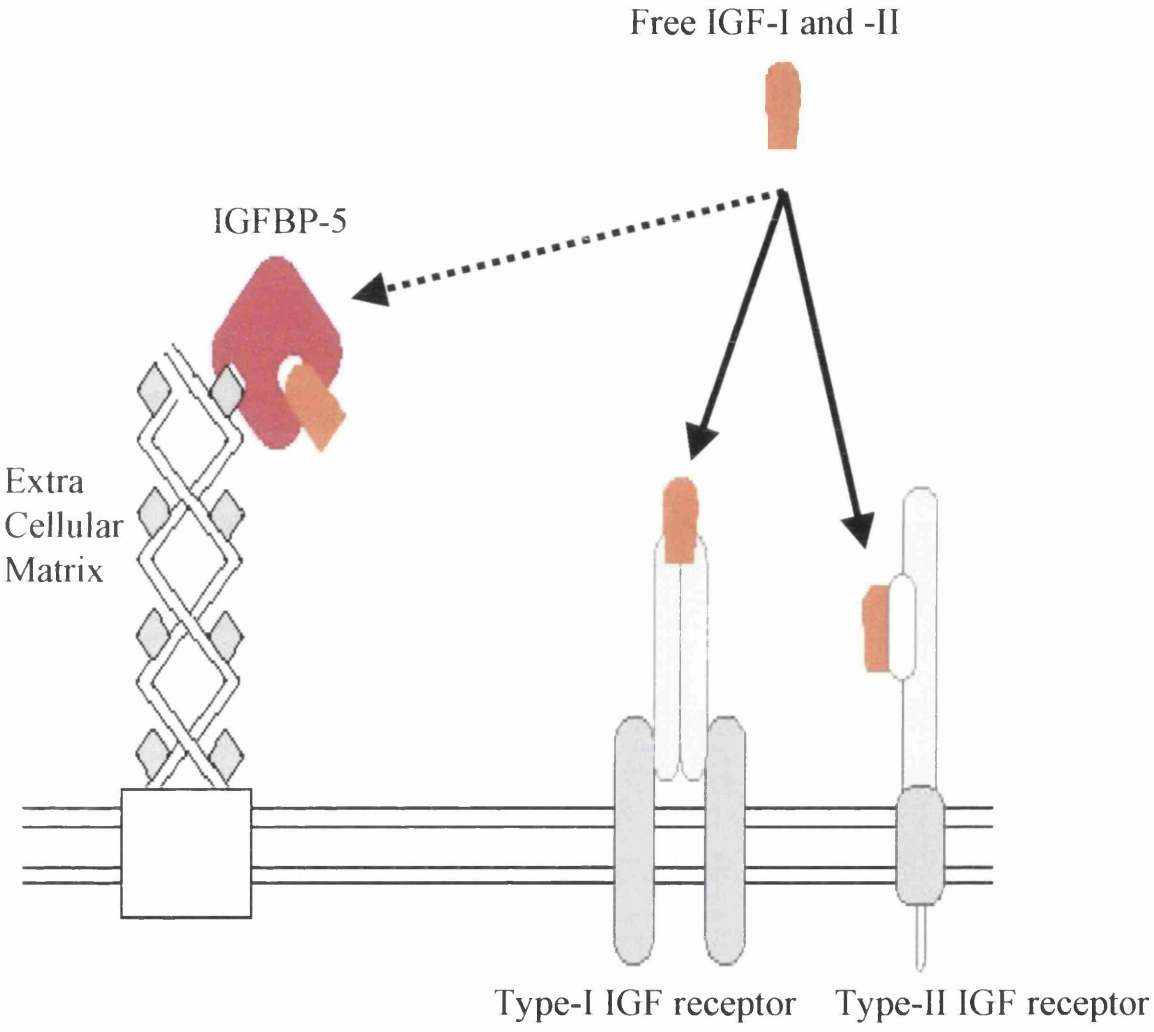


Figure I.7 Pro-apoptotic model of IGFBP-5

that apoptosis of these cells involves IGFBP-5 mediated cell signalling (Andress, 1995). In the involuting mammary gland, the majority of IGFBP-5 is found in the intact form, although a number of active proteolytic enzymes have been identified there (Tonner *et al*, 1997).

Finally, the plasminogen activation system is also involved in tissue remodelling and apoptosis, and this provided a further route for IGFBP-5 action. IGFBP-5 has been shown to bind plasminogen-activating inhibitor-I (PAI-I), through the basic amino acid region in the C-terminus of the binding protein (201-218) (Nam *et al*, 1997). More recently, it was shown that IGFBP-5 also binds to the milk protein αS2-casein and, in particular, to its dimeric form (Tonner *et al*, 2000). Dimeric αS2-casein has also been shown to bind to both plasminogen and t-PA (tissue-type plasminogen activator), and this binding results in the enhanced activity of t-PA, thereby enhancing the conversion of plasminogen to plasmin (Heegaard *et al*, 1997). Plasmin plays a central role in cleaving a number of pro-enzymes, such as procollagenases, and thereby initiating the degradation of the ECM at the end of lactation when extensive tissue remodelling occurs (Matrisian, 1990). Therefore, the close physical proximity of IGFBP-5 and components of the plasminogen system on the casein micelle suggest a possible functional interaction, supported by the observation that IGFBP-5 binds to PAI-I (Nam *et al*, 1996). As PAI-I binds to t-PA and inhibits its actions, it is conceivable that IGFBP-5 plays a dual role in tissue remodelling/apoptosis of the mammary gland: 1) IGFBP-5 activates cell death by sequestration of IGF-I, and 2) IGFBP-5 activates plasminogen cleavage by sequestering PAI-1 (Tonner *et al*, 2000). In support of this, both prolactin and GH inhibit t-PA activity and plasminogen activation in the involuting mammary gland (Tonner *et al*, 2000). In conclusion, IGFBP-5 may have a central role in co-ordinating cell death and tissue remodelling processes.

I.4.3 Stimulation of IGFs action

IGFBP-1. As reviewed in Clemmons (1997), potentiation of IGF effects by IGFBP-1 is generally observed when the non-phosphorylated isoform of the binding protein is used, or when it is added at low concentrations (Elgin *et al*, 1987; Busby *et al*, 1988). IGFBP-1 enhanced IGF-I, but not IGF-II, stimulated proliferation in human keratinocytes and skin fibroblasts (Kratz *et al*, 1992)

and increased DNA synthesis in human, mouse embryo fibroblasts (Schuller *et al*, 1993). In contrast to the aforementioned inhibitory effect of IGFBP-1 in pSMC, this binding protein was reported to increase the IGF-I-stimulated ^{3}[H]-thymidine uptake 4.8-fold, while IGFBP-2 increased this IGF action 2.2-fold under the same conditions (Bourner *et al*, 1992).

IGFBP-3. As reviewed in Clemmons (1997), stimulation of IGF action by IGFBP-3 is mainly thought to involve sequestering IGFs to cell surfaces (Baxter, 1988; Conover *et al*, 1992). When either recombinant human glycosylated or non glycosylated IGFBP-3 was incubated with bovine fibroblasts, it associated to the cell monolayer, and provided an additional low affinity binding site for ^{125}I-IGF-I (Conover, 1992). After 72h pre-incubation of the cells with IGFBP-3, aminoisobutyric acid (AIB) and thymidine uptake stimulated by IGF-I, insulin and [QAYL]IGF-I (which has full affinity for IGF-I-R and reduced affinity for IGFBP-3) were potentiated. Cell responsiveness to the growth factors correlated with their affinity for IGF-I-R, but not their affinity for IGFBP-3. It could be hypothesized that IGFBP-3 localizes IGF-I on the cell membrane and slowly releases it to the receptor, thus preventing the down regulation of IGF-I-R activity that is induced by an excess of IGF.

The human colon cancer cell lines COLO205, HT29 and SW620 are known to secrete IGF-II and IGFBPs. Michell *et al* (1997) characterized the sensitivity of these cell lines to exogenous IGF-I and examined the effects of their autocrine IGFBPs on these responses. Cells cultured in serum-free medium were treated with 1-100ng/ml IGF-I, or des[1-3]IGF-I (a truncated IGF-I which does not appreciably bind IGFBPs), and DNA synthesis was determined by 24h incorporation of ^{3}[H]-thymidine. Experiments were repeated in the presence of 24h cell-conditioned media containing endogenous IGFBPs. In all three cell lines, cell-conditioned media reduced the sensitivity to IGF-I but not to des[1-3]IGF-I suggesting that IGFBPs in the cell-conditioned media of colon cells inhibit IGF-I action. IGFBP-4 was identified in all cell lines and IGFBP-2 from the COLO205 and SW620 cell lines, but not the HT29 cells. No IGFBP-3 was identified in the 24h cultured conditioned media, but IGFBP-3 was found in the cell membrane of all three cell lines. Interestingly, when endogenous secreted IGFBPs were removed, the cell lines were consistently less sensitive to des[1-3]IGF-I suggesting that IGFBP-3 associated with the cell layer enhances responses to IGF-I. In addition, Giannini *et al* (1999) showed that IGFBP-3 is involved in growth

regulation of human glomerular endothelial cells (GENC). When they added IGFBP-3 antibody to the culture media, cell proliferation was significantly reduced, whereas recombinant human IGFBP-3 alone had no effect on these cells. However, after 48h pre-incubation with IGFBP-3 the IGF-I stimulated GENC growth was increased, suggesting that IGFBP-3 could potentiate the IGF-I induced GENC proliferation.

Both IGFBP-3 and transforming growth factor-beta (TGF-β) have been separately shown to have cell-specific growth-inhibiting or growth-potentiating effects (Oh *et al*, 1995). TGF-β stimulates IGFBP-3 mRNA and peptide expression in several cell types, and TGF-β-induced growth inhibition and apoptosis have been shown to be mediated through the induction of IGFBP-3 (Oh *et al*, 1995). Cohen *et al.* (2000) reported that TGF-β treatment of airway smooth muscle (ASM) cells resulted in a 10- to 20-fold increase in IGFBP-3 mRNA and protein expression. Addition of either IGFBP-3 or TGF-β to the culture medium also resulted in an approximately two-fold increase in cell proliferation. Coincubation of ASM cells with IGFBP-3 antisense oligomers as well as with an IGFBP-3 neutralising antibody blocked the growth induced by TGF-β. These findings suggest that IGFBP-3 is capable of mediating the growth stimulatory effect of TGF-β in ASM cells.

IGFBP-5. As reviewed in Clemmons (1997), stimulatory effects of IGFBP-5 have been reported in various cell lines. These effects may be dependent upon the form of IGFBP-5 present. For example, ECM-associated IGFBP-5 enhanced human fibroblast growth in response to IGF-I treatment, while the binding protein in the conditioned medium was degraded to a 21kDa fragment that lacked any potentiating effects (Jones *et al*, 1993b). In bone cells, as with smooth muscle cells, IGFBP-5 has been reported to have both cell proliferation and differentiation promoting effects (reviewed by Clemmons, 1997). In human osteoblast-like cells both IGFBP-5 and its carboxy-truncated 23kDa fragment enhanced IGF-I and IGF-II stimulated mitogenesis (Andress *et al*, 1993). IGFBP-5 also appears to stimulate proliferation of these cells by both IGF-dependent and independent pathways (Andress and Birnbaum, 1992). During the differentiation process in MC3T3-E1 mouse osteoblasts, IGFBP-5 secretion increases in the initial cell proliferation period and it reaches its peak at the start of differentiation, after which it declines (Thraikill *et al*, 1995b). In contrast, it has been noticed that in rat osteoblasts, growth factors which stimulated cell

proliferation, such as FGF, TGF-β and PDGF, decreased the expression of IGFBP-5, IGF-I and IGF-II (Canalis and Gabbitas, 1995), while factors that stimulated cell differentiation, (namely IGFs and retinoic acid), increased IGFBP-5 expression (Conover and Kiefer, 1993; Dong and Canalis, 1995; Gabbitas and Canalis, 1998). Cortisol, which is an inhibitor of multiple parameters of bone formation, including cell proliferation, reduced IGFBP-5 expression and its deposition onto the ECM (Gabbitas *et al*, 1996).

Recently Kanatani *et al.* (2000) reported the effects of IGFBP-5 on osteoclastic activity and osteoclast formation. These observations demonstrated both IGF-dependent and IGF-independent regulation of IGFBP-5. IGFBP-5 significantly stimulated pit formation by pre-existent osteoclasts in mouse bone cell cultures and its stimulatory effect was completely blocked by an IGF-I antibody (Ab). However, IGFBP-5 did not affect the bone-resorbing activity of isolated rabbit osteoclasts. A direct effect of IGFBP-5 on osteoclast precursors was also examined and it was shown that IGFBP-5 stimulated osteoclast-like cell formation from osteoclast precursors in a dose dependent manner, irrespective of the presence of IGF-I Ab. The present data indicate that IGFBP-5 stimulates bone resorption both by stimulation of osteoclast formation in an IGF-I-independent fashion and by IGF-I-dependent activation of mature osteoclasts, possibly via osteoblasts.

As with bone cells, there are contrasting results published suggesting that IGFBP-5 potentiates either the proliferating or the differentiating effect of IGF peptides on smooth muscle cells (SMC). In porcine SMC transfected with IGFBP-5 cDNA the expressed protein accumulated in the ECM more abundantly than in the control cells and it potentiated the IGF-I stimulation of DNA synthesis. Conversely, the transfection of pSMC cells with IGFBP-5 mutants which have reduced ECM binding affinity, resulted in an inhibition of IGF-I stimulated DNA synthesis (Parker *et al*, 1998).

Similar results in terms of potentiation of IGF stimulated ^{3}[H]-thymidine uptake were obtained in SMC when monolayers were incubated for 48h with IGFBP-5. (Clemmons, 1998). It was suggested that, in order to achieve an IGF enhancing effect, IGFBP–5 had to be proteolyzed into a 21kDa fragment, which retained the ability to associate to the ECM but had greatly reduced affinity for IGFs. This theory is further strengthened by the observation that a proteolysis resistant

IGFBP-5 mutant had an IGF inhibitory effect in the same culture conditions (Duan and Clemmons, 1998). In this latter study it was shown that IGFBP-5 and IGF expression is high in proliferating cells and declines when cells reach confluence. In contrast, other authors report that in L6A1 muscle cells (Ewton *et al*, 1998) and in C2 mouse myoblasts (Rousse *et al*, 1998) IGFBP-5 expression increases during cell differentiation. In L6A1 cells IGFBP-5 expression was higher in differentiating cells and the binding protein showed an inhibitory effect on IGF stimulated proliferation and a potentiating effect on cell differentiation stimulated by IGF-I but not IGF-II (Ewton *et al*, 1998).

I.4.4 IGF-independent functions

The discovery of IGF-independent modulation of growth by IGFBPs provided evidence for the presence of specific cell-surface IGFBP receptors and added a further layer of complexity to the IGF axis. These actions are more accurately described as IGF-I-R independent, because they may be modulated by IGF binding without requiring the presence of the IGF-I-R. Such actions are likely to involve structural domains of IGFBPs that are distinct from the IGF-binding determinants.

IGFBP-1 The first characterized of these domains is the Arg-Gly-Asp (RGD) integrin-binding motif present in the carboxy-terminu

s of IGFBP-1 and –2, but not in any other IGFBP. The interaction of the C-terminal RGD sequence of IGFBP-1 with the cellular $\alpha_5\beta_1$ integrin (fibronectin receptor) on the cell surface has been described (Jones *et al*, 1993a). Post-translational modifications might influence this binding as it has been reported that only the phosphorylated form of IGFBP-1 can associate with the cell surface (Chan and Spencer, 1997). Integrins that have been considered principally as adhesion molecules are now increasingly reported to be involved in cellular signaling events (reviewed by Baxter, 2000). The integrin α subunit is responsible for ligand specificity, while the β subunit determines the signal specificity, which means that the stimulation by different ligand molecules can lead to the same cellular response.

Cell migration is a complex phenomenon resulting from the interaction between several growth factors, the cells and the extracellular matrix (Gockerman *et al*, 1995). Therefore a

combination of factors, including the integrin type expressed on the cell surface and the cellular substratum, can affect the ability of a molecule to stimulate cell migration. IGFBP-1 stimulates cell migration in CHO cells (Jones *et al*, 1993a) and in pSMC cells seeded on fibronectin coated plates (Gockerman *et al*, 1995). The RGD sequence appears to be essential for this stimulation to occur as demonstrated by the inhibition of the IGFBP-1-stimulated cell migration by a competitive RGD peptide or by the inability of a WGD-IGFBP-1 mutant to stimulate cell migration. It has been shown that fibronectin inhibits cell migration in CHO cells overexpressing $\alpha_5\beta_1$ integrin (Giancotti and Ruoslahti, 1990) and although it is involved in cell adhesion, it does not facilitate cell migration in pSMC cells (Clark, 1993). Therefore IGFBP-1 stimulation of cell migration could result from its competition with fibronectin to bind $\alpha_5\beta_1$ integrin. (Gockerman *et al*, 1995).

IGF-I and II alone are also potent stimulators of pSMC cell migration on fibronectin coated plates (Gockerman *et al*, 1995). In this case, IGFBP-1 was shown to exert an inhibitory effect on IGF-stimulated migration by antagonizing the binding of the growth factors to IGF-I-R. IGF-I also promotes pSMC cell migration on vitronectin coated plates, but in this case both the stimulation of the IGF-I-R by IGF-I and the ligand occupancy of $\alpha_v\beta_3$ integrin (vitronectin receptor) appear to be necessary (Jones *et al*, 1995). The addition of either IGFBP-1 to antagonize IGF/IGF-I-R interaction or $\alpha_v\beta_3$ integrin competitors (such as echistatin and kistrin) inhibits this IGF-I-stimulated cell migration (Jones *et al*, 1995).

IGFBP-3 and -5. Apart from IGFBP-1, the only other proteins for which IGF-I-R independent functions have been demonstrated are IGFBP-3 and –5.

Study of Hs578T human breast cancer cells has demonstrated that IGFBP-3 is the major IGFBP species secreted and that it associates with cell surface membranes (Oh *et al*, 1992). This work suggested that IGFs may specifically regulate the concentration of IGFBP-3 in Hs578T conditioned medium (CM) through non-receptor mediated dissociation of cell surface-associated IGFBP-3. The same group presented work which suggested the existence of a specific IGFBP-3 receptor in Hs578T cells (Oh *et al*, 1993a-c). They demonstrated that both exogenous and endogenous IGFBP-3 binds to the Hs578T cell surface and inhibits cell proliferation stimulated by IGF-I, but not that by IGF analogs with reduced affinity for IGFBP-3. By cross-linking ^{125}I-IGFBP-3 to the cell monolayer and subsequently immune precipitating the cell lysate with antibodies raised

against IGFBP-3, three cell surface proteins (20, 26 and 50 kDa) were identified with IGFBP-3 binding properties. Therefore, the authors proposed that the IGFBP-3 inhibitory effect on cell proliferation could be mediated through a specific IGFBP-3 receptor.

In addition, it was shown that substances that inhibited Hs578T cell growth, such as TGF-β_2 (transforming growth factor), anti-estrogens and retinoic acid, induced an increase of IGFBP-3 expression (Oh *et al*, 1995). TGF-β-induced growth arrest was also significantly reversed by transfecting the cells with antisense IGFBP-3. Furthermore, IGF-II and Leu^{27} IGF-II (which has wild-type affinity for IGFBP-3, but reduced affinity for IGF-I-R) were able to reverse TGF-β stimulated growth suppression, whereas treatment with an IGF-II analogue (which has reduced affinity for IGFBP, but wild type affinity for IGF-I-R) could not. These data indicate that the mechanism by which transforming growth factor-β (TGF-β) supresses cell growth cannot simply be explained by an increase in IGFBP-3 and subsequent sequestration of IGFs from IGF-I-R, and suggests that IGFBP-3 acts in an IGF-independent fashion (Oh *et al*, 1995). Related to this, Leal *et al*. (1997) suggested that the type V TGF-β receptor is a putative cell surface associated IGFBP-3 receptor in mink lung epithelial cells. They also found IGFBP-4 and –5 (less potent than IGFBP-3) are additional ligands for the type V TGF-β receptor, which mediates the growth inhibitory response through signaling pathways distinct from that mediated by the TGF-β heterocomplex (Leal *et al*, 1999). Further support for a specific IGFBP-3 receptor came from a study where it was shown that IGF-I-R-negative mouse fibroblasts displayed lower growth rate compared to the controls when transfected with human IGFBP-3 (Valentinis *et al*, 1995). Moreover, several reports have shown that IGFBP-3 fragments derived by limited plasmin proteolysis were able to inhibit IGF-stimulated mitogenesis and even the mitogenic effect of insulin, despite their reduced affinity for IGF (Oh, 1997).

In the prostate cell line, PC-3, IGFBP-3 fragments derived by plasmin digestion displayed the opposite biological effect to those described above (Oh, 1997). Intact IGFBP-3 was able to increase cell proliferation and a fragment of IGFBP-3 containing residues 1-169 ($^{1\text{-}169}$IGFBP-3) was almost three times more effective than the wild type molecule. This effect is probably due to a modulation of endogenous IGF-II-induced cell proliferation as it is markedly reversed by an anti-IGF-I-R monoclonal antibody (αIR-3). In contrast, a $^{1\text{-}95}$IGFBP-3 fragment, derived from further

digestion of the 1-169 peptide, reduced cell proliferation to 50% of basal levels. This fragment which does not bind IGF-II, was able to reverse IGF-II induced cell proliferation even when the accessibility of IGF-I-R was blocked with the addition of αIR-3 (Angelloz-Nicoud *et al*, 1998). This data confirms previous work which described the growth inhibitory effect of the $^{1\text{-}95}$IGFBP-3 fragment in mouse fibroblasts with targeted disruption of IGF-I-R gene (Mohseni-Zadeh and Binoux, 1997). Therefore, these data suggest that $^{1\text{-}169}$IGFBP-3 has IGF-I-R dependent effects on cell proliferation, whereas $^{1\text{-}95}$IGFBP-3 has an inhibitory effect on cell proliferation through an IGF-I-R independent manner.

Yamanaka *et al.* (1999) evaluated IGFBP-3 binding sites on breast cancer cell membranes by competitive binding studies with IGFBP-1 through –6 and various forms of IGFBP-3, including synthetic IGFBP-3 fragments. Among the IGFBPs, only IGFBP-5 showed weak competition, indicating that IGFBP-3 binding to breast cancer cell surfaces is specific. This was confirmed by showing that synthetic IGFBP-3 peptides containing IGFBP-3 glycosaminoglycan-binding domains competed only weakly for IGFBP-3 binding to the cell surface. Rat IGFBP-3 was 20-fold less potent in its ability to compete with human IGFBP-3, as well as 10- to 20-fold less potent for cell growth inhibition than human IGFBP-3. When various IGFBP-3 fragments were evaluated for affinity for the IGFBP-3 receptor, only those fragments that contain the midregion of the IGFBP-3 molecule were able to inhibit IGFBP-3 binding, indicating that the midregion of the IGFBP-3 molecule is responsible for binding to its receptor (Yamanaka *et al*, 1999).

An alternative method of interaction between the cells and IGFBPs has recently been described for IGFBP-3 and -5 when they were shown to translocate to the cell nucleus. Using immunofluorescence, IGFBP-3 was detected in the nucleus of proliferating human keratinocytes and laser scanning confocal microscopy confirmed that the binding protein was present in the nucleus and not in the perinuclear spaces (Wraight *et al*, 1998). Nuclear uptake of fluorescently labeled IGFBP-3 and 5 was demonstrated in proliferating human breast cancer cells after 90 minutes, whereas no nuclear uptake was observed after the addition of an equivalent amount of labeled IGFBP-1 and 2, although all labeled proteins were shown to associate to the cell membrane (Schedlich *et al*, 1998). Both IGFBP-3 and 5 contain a NLS sequence (nuclear localization signal) inside the C-terminal 18 residue sequence (215-232 for IGFBP-3 and 201-218 for IGFBP-5) that is

also involved in heparin/GAGs and ALS binding (Radulescu, 1994). Based on this structural study of IGFBP-3, the same group used immunocytochemistry to identify the nuclear localization of IGFBP-3 in a lung cancer cell line where IGFBP-3 antibodies specifically stained the nuclei (Jaques *et al*, 1997). This finding suggests the possibility that nuclear IGFBP-3 is functional and involved in the pathogenesis of lung cancer.

The NLS motif is a bipartite nuclear targeting sequence found in many nuclear proteins and consists of 2 basic residues followed by an interval of 10-11 amino acids and then at least 3 basic residues within the next 5 positions. Schedlich *et al.* (1998) reported that substitution of the (^{228}KGRKR) IGFBP-3 sequence with the corresponding (MDGEA) IGFBP-1-derived sequence abolishes nuclear uptake (Schedlich *et al*, 1998). IGFBP-3 nuclear localization does not seem to be affected by lysosomotropic inhibiting agents nor by microtubule disrupting agents, suggesting the internalization and translocation occurs through an alternative pathway (Schedlich *et al*, 1998). Furthermore, Schedlich *et al.* (2000) describes the mechanism for nuclear import of IGFBP-3 and IGFBP-5. In digitonin-permeabilised cells where the nuclear envelope remains intact, nuclear translocation of wild type IGFBP-3 appears to occur by a nuclear localisation sequence (NLS)-dependent pathway mediated principally by the importin-β, nuclear transport factor, and requiring both ATP and GTP hydrolysis. In cells where both the plasma membrane and nuclear envelope were permeabilized, wild-type IGFBP-3, but not the mutant form (MDGEA), accumulated in the nucleus, implying that the NLS was also involved in mediating binding to nuclear components. By fusing wild type and mutant forms of NLS sequences (IGFBP-3 [215-232] and IGFBP-5 [201-218]) to the green fluorescent protein, they identified the critical residues of the NLS necessary and sufficient for nuclear accumulation. Using western-ligand binding assays, wild-type IGFBP-3 and IGFBP-5, but not an NLS mutant form of IGFBP-3, were shown to be recognised by importin-β and the alpha/beta heterodimer, but only poorly by importin alpha. These results suggest that the NLS within the C-terminal domain of IGFBP-3 and IGFBP-5 are required for importin-β-dependent nuclear uptake, and probably also accumulation through mediating binding to nuclear components (Schedlich *et al*, 2000). Finally, although IGFBP-3 preferentially binds to the cell surface, it can also associate to ECM, although less potently in comparison with IGFBP-5 (Jones *et al*, 1993b; Imai *et al*, 1997).

Andress and Birnbaum (1992) reported that a purified 23kDa fragment of IGFBP-5 from cultured human osteoblast-like cells enhanced mitogenesis. This effect was unique from other binding proteins in not requiring a preincubation period or serum co-factors, and furthermore, the mitogenesis was stimulated by endogenous IGFBP-5 without exogenous or endogenous IGFs. Therefore, the authors suggested that this was an IGF-independent action of IGFBP-5 (Andress and Birnbaum, 1992). Similar results reported that IGFBP-5 potentiated bone cell proliferation even in the absence of IGFs, suggesting an IGF-independent mechanism involving IGFBP-5-specific cell surface binding sites (Mohan *et al*, 1995). Moreover, in cultured mouse osteoblast cells, recombinant carboxyl-truncated IGFBP-5^{1-169} has been demonstrated to be able to bind to osteoblast monolayers and stimulate mitogenesis without exogenous IGF-I (Andress *et al*, 1993). While it was proposed that this intrinsic mitogenic action of truncated IGFBP-5 was mediated by cell surface binding, the exact mechanism of IGFBP-5 binding to osteoblasts was not determined. Subsequently, the same author has presented evidence for the existence of a 420kDa osteoblast cell membarane protein that acts as an "IGFBP-5 receptor" (Andress, 1995). This work described the purification of the putative receptor using IGFBP-5 affinity chromatography and also demonstrated that IGFBP-5 binding to the receptor was not competed by solubilized heparan sulfate proteoglycan. In conclusion, the author suggests that this 420 kDa membrane protein may be involoved in receptor-mediated signal transduction, since earlier studies demonstrated that IGFBP-5^{1-169} directly stimulates mitogenesis in mouse osteoblasts (Andress *et al*, 1993). In support of this, the same author has more recently presented evidence for serine phosphorylation of the 420 kDa protein as a result of binding by intact IGFBP-5, IGFBP-5^{1-169} or IGFBP-$5^{201-218}$ peptides (Andress, 1998).

I.5 Aims of study

Recent work from our laboratory has addressed the role of IGFBP-5 in the developing mammary gland. First, we have observed up-regulation of IGFBP-5 expression during apoptosis of mammary epithelial cells in the involuting mammary gland (Tonner *et al*, 1995 and 1997). As discussed above, a pro-apoptotic model has been proposed to account for this effect of IGFBP-5. In this model, IGFBP-5 sequesters IGFs to casein micelles, prevents growth factors/receptor interaction, and thereby initiates apoptosis. Second, an IGF-independent role of IGFBP-5 has been suggested in remodelling of ECM in the mammary gland via up-regulation of the plasminogen system. Here IGFBP-5 is proposed to sequester PAI-I to casein micelles, thereby enhancing tissue t-PA levels, which, in turn, results in production of the ECM degrading enzyme, plasmin. In order to increase our understanding of these potential IGFBP-5 functions, the work described in this thesis was focussed on the relationship between the binding protein's structure and its important molecular interactions.

The aims of this study were:

1) To assess the separate contributions of the N- and C-terminal domains of IGFBP-5 with respect to IGF binding.

2) To identify specific amino acids within the IGFBP-5 protein sequence that are important IGF binding determinants, with the aim of producing a mutant that cannot bind IGFs.

3) Based on the pro-apoptotic model of IGFBP-5 action, to further investigate sequences in the binding protein involved in ECM binding, using the ECM glycosaminoglycan heparin as a model. Furthermore, to produce an IGFBP-5 mutant which cannot bind heparin/ECM.

4) To produce a variety of IGFBP-5 mutants that are defective in important molecular interactions, which can then be used to test the biological functions of the binding protein in appropriate cell systems.

Chapter II. Materials and Methods

General laboratory chemicals and reagents were supplied by BDH (Poole, Dorset, UK) and by Fisher Scientific (Loughborough, Leicestershire, UK). Unless specified otherwise, most of the other chemicals were from Sigma (Poole, Dorset, UK). Cell culture media and supplements were supplied by Gibco BRL, Life Technologies (Paisley, UK) or Sigma, and molecular biology reagents were from Boehringer Mannheim (East Sussex, UK), Promega (Southampton, UK) or New England Biolabs (Hertfordshire, UK). Cell culture plasticware was supplied by Costar (Bucks, UK) or Greiner Labortechnik (Stonehouse, Glos, UK). Water (tissue culture grade) was from Life Technologies, otherwise double distilled tap water was used. Unless otherwise stated, all centrifugations of Eppendorf tubes were carried out with bench top microfuge (MSE microcentaur), while centrifugations necessary for cell culture routine mantainance and experimental work were conducted in a MSE mistral 2000 centrifuge (MSE Loughborough, Leicestershire, UK).

II.1 Solutions/Buffers and basic methodologies

II.1.1 Solutions and buffers

A. Nucleic acid work

10 X TBE (Tris Borate EDTA)

1M Tris-HCl, 0.9M Boric acid, 0.01M EDTA, pH 8.4.

10 X TAE (Tris Acetate EDTA)

0.4M Tris, 0.2M Na acetate, 0.01M EDTA, pH7.2 with glacial acetic acid.

5 X Orange G (electrophoresis loading DNA sample buffer)

100mM EDTA, 20% *(w/v)* Ficoll, 0.1% (*w/v*) Orange G.

TE buffer

10mM Tris-HCl, pH8.0, 1mM EDTA, Autoclaved.

LB (Luria-Bertaini) medium

1% *(w/v)* Tryptone (Oxoid, Hampshire, UK), 0.5% *(w/v)* Yeast extract, 0.5% *(w/v)* Sodium Chloride, pH 7.5, Autoclaved.

LB agar plate

LB medium, 1.5% *(w/v)* Agar (Oxoid, Hampshire, UK), pH 7.0. Autoclaved and with addition of appropriate antibiotics.

Antibiotic stock solutions

Ampicillin 125mg/ml in H_2O, Tetracycline 12.5mg/ml in 80% (*v/v*) ethanol, Kanamycin 10mg/ml in H_2O, Gentamycin 7mg/ml in H_2O.

B. Baculovirus expression system

Formyl buffered Acetone (4ml)

1.2ml Phosphate buffered saline, 1ml 37% *(v/v)* formaldehyde solution, 1.8ml Acetone.

Bacmid selection LB plates

Autoclaved LB agar, 50μg/ml Kanamycin 7μg/ml, Gentamycin 10μg/ml, Tetracycline 40μg/ml, IPTG 200μg/ml, 5-bromo-3-indolyl-β-$_D$-galactopyranoside.

C. Protein purification

Equilibrium buffer

0.5M Sodium Chloride, 50mM Tris-HCl, pH 6.5.

Basic washing buffer

0.1M Tris-HCl, 0.5M Sodium chloride, pH 8.5.

Acidic washing buffer

0.1M Sodium Acetate, 0.5M Sodium Chloride, pH 4.5.

Elution buffer

0.5% *(v/v)* Acetic acid, pH 3.0.

Concentrated Tris buffer

2M Tris base.

Column strorage buffer

50mM Tris-HCl, 0.5M Sodium Chloride, pH 7.4, 0.01% *(w/v)* Sodium azide.

D. Iodination

Radio Immuno Assay (RIA) buffer per litre

30mM Sodium Chloride, 60mM Sodium Phosphate, 0.25mM Thimerosal, 0.5% *(w/v)* BSA, pH 7.4.

Sodium Phosphate solution (Pi)

0.5M Sodium Phosphate, pH 7.0.

E. Protein electrophoresis and blotting

1 X Sample buffer (SDS-page protein gel loading buffer)

0.8M Tris-HCl, 2% *(w/v)* SDS, 10% *(v/v)* Glycerol, 1.2% *(w/v)* Bromophenol blue, pH 6.8, Adding 5% *(v/v)* β-mercaptoethanol for the reducing gel.

10% SDS

10% *(w/v)* Sodium dodecyl sulfate in distilled H_2O.

10 X Running buffer (protein gel electrophoresis buffer)

0.25M Tris base, 2M Glycine, 1% *(w/v)* SDS.

10 X Blotting buffer (Gel to membrane transfer buffer)

0.25M Tris base, 2M Glycine, 20% *(v/v)* methanol.

10 X TBS (Tris buffered saline)

0.1M Tris-HCl, 530mM Sodium Chloride, pH 7.6, + 0.5% (v/v) Tween-20 for TBS-Tween.

10 X PBS (Phosphate buffered saline) per litre

0.1M Sodium Chloride, 10mM di-Sodium hydrogen orthophosphate, 10mM Sodium dihydrogen orthophospate, pH 7.0, + 0.5% *(v/v)* Tween-20 for PBS-Tween.

F. Charcoal binding solution phase assay

1 X Charcoal binding solution assay buffer

50mM Tris-HCl, 0.5% *(w/v)* BSA, pH 7.4.

1 X Charcoal binding solution

1% (*w/v*) activated charcoal (Sigma), 50mM Tris-HCl, 0.5% *(w/v)* BSA,pH 7.4, 0.2% (*w/v*) Protamine sulfate.

II.1.2 Preparation of Gels

A. DNA Gels

Agarose gels

1% *(w/v)* high gelling temperature agarose (Promega) in 1xTBE buffer and 0.5μg/ml of ethidium- bromide (Biorad, Hertfordshire,UK). Size markers were purchased from Promega; these were λHindIII (23130, 9416, 6557,4361,2322, 2027, 564 and 125 base pairs) and $\phi\chi174$ (1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72 base pairs).

B. Protein Gels

4% Stacking gel

0.25M Tris-HCl, 4% *(w/v)* total acrylamide (ratio 37.5:1 – bisacrylamide), 0.1% *(w/v)* SDS, 5mM AMPS, 0.1% *(v/v)* tetramethylethylenediamine (TEMED).

12.5% Running gel

0.375M Tris-HCl, 12.5% *(w/v)* total accrylamide (ratio 37.5:1 – bisaccrylamide), 0.1% *(w/v)* SDS, 5mM AMPS, 0.05% *(v/v)* TEMED.

16% Running gel

0.375M Tris-HCl, 16% *(w/v)* total accrylamide (ratio 37.5:1 – bisaccrylamide), 0.1% *(w/v)* SDS, 5mM AMPS, 0.05% *(v/v)* TEMED.

II.1.3 Cell lines

A. Bacterial Cell lines

The *Escherichia coli* (E. coli) strains **JM109** (Yanisch-Perron *et al*, 1985) and **JM110** (Yanisch-Perron *et al*, 1985) were used for basic DNA manipulation.

ES1301*mutS* cells (Promega), a mismatch repair minus strain of *E. coli* (Siegel *et al*, 1982), were used for the site-directed mutagenesis. *MutS* strain prevents repair of the newly synthesized unmethylated strand (Siegel *et al*, 1982; Kramer *et al*, 1984), leading to high mutation efficiencies.

DH10Bac (Gibco BRL) strain was used to produce recombinant baculovirus molecules for the expression of eukaryotic proteins (Luckow *et al*, 1993). This cell contains the parent bacmid pMON14272 and the helper plasmid pMON7124. The parent bacmid contains a mini-F replicon, the kanamycin resistance gene, an attTn7 site and the *lacZα* complementation factor. The helper plasmid contains the tnsABCD region which supplies the transposition proteins required for insertion of the mini-Tn7 from the donor plasmid into its target site on the parent bacimd.

B.Insect Cell line

Sf9 A clonal isolate of the *Spodoptera frugiperda* cell line IPLB-sf21-AE, was used for expressing recombinant baculovirus and proteins. Sf9 has been originally established from ovarian tissue of the fall armyworm (Vaughn *et al*, 1977), and selected for its high susceptibility to baculovirus infection.

Insect cell culture. We received as a gift from Life Technologies a frozen vial of cells already adapted to serum free conditions. Both wt and mutant IGFBPs were expressed in Sf9 cells with serum free medium (SFM), protein free (SF900-II Gibco) conditions for several reasons. Firstly, as IGFBPs are secreted proteins, we intended to recover the expressed protein directly from the supernatant of cultures insect cells grown in suspension. The manufacturing company (Life Technologies) that commercialises the Bac-to-Bac expression system claims that, in serum free conditions, the recombinant secreted protein represents the major protein species in the insect cell supernatant, so that further purification might not be required. Secondly, serum bovine IGFBPs would contaminate the recombinant protein preparation and the separation between rat and bovine IGFBPs would require a laborious reverse-phase chromatography step. Finally, protease activity contained in the serum could potentially degrade the expressed protein.

Sf9 insect cell culture was initiated directly in suspension, thawing $1.7x10^7$ cells in 40ml of SF900-II medium (without addition of antibiotics or antimycotics) in a 50ml spinner flask (Techne, Cambridge, UK). Cells were routinely grown at 27°C, with stirring at 75rpm using a Techne MCS-104S biological stirrer placed inside the incubator. Cell viability was checked every day with Trypan blue staining (0.5% *w/v* Trypan in PBS, mixed at 1:2 with the cells). Cell viability was consistently over 99%, with the larger cell clusters depositing on the bottom of the flask. These were routinely removed by aspiration. Cell density was maintained between 0.5 and 2 x 10^6cells/ml.

It is recommended to use SFM-adapted cells only for a low number of passages (12-15), as culture of insect cells in SFM in suspension has been reported to decrease cell viability and foreign protein expression (Clemm, 1994). For this reason we made frozen stocks of early passage cells. Cells were harvested in mid log phase with a viability >97%. After 5min of centrifugation at 1000rpm, the cells were resuspended at a density of 1-2 x10^7cells/ml in medium containing 7.5% *v/v* DMSO and 92.5% of a 1:1 mix of fresh and cell-conditioned SF900-II medium. Vials were

wrapped in cotton wool, placed in a polystyrene box, stored at –70°C overnight and then transferred to liquid nitrogen.

C. Mammalian Cell lines

C2 myoblast cell line was derived from thigh muscle of C3H mice (Yaffe and Saxel, 1997). This cell line is used as a biochemical parameter of differentiation by monitering activity of creatine kinase (Yaffe and Saxel, 1997). The cell line was kindly donated by Dr. Claire Stewart (University of Bristol, Bristol, UK) and used to test the biological function of IGFBP-5. This cell line expresses IGFBP-5 mRNA and protein during its terminal differentiation. Furthermore, IGFBP-5 appears to be the only IGFBP produced by C2 cells and by another rapidly fusing muscle cell line, F3 azamyoblast clone b (James *et al*, 1993; Rotwein *et al*, 1995; Roussse *et al*, 1998). Cells were cultured at 37°C on 0.2% *w/v* gelatin (type A: from porcine skin, Sigma) coated T75 flasks (Corning) with DMEM L-glutamax medium (Gibco) containing 10% *v/v* foetal bovine serum (Gibco) and 10% *v/v* new born calf seurm (Gibco). At confluence cells were washed with PBS and detached from the plastic surface by a few minutes incubation at 37°C in the presence of 3-7ml of X1 Trypsin-EDTA solution (0.5mg/ml Trypsin and 0.2mg/ml EDTA, obtained by diluting x10 concentrated solution with HBSS). 15-20ml of 10% FBS F12 medium were added to inactivate trypsin, then cells were harvested in 50ml centrifuge tubes and centrifuged at 1000rpm for 5min. Supernatant was discarded and cell pellets resuspended in complete medium.

Primary goat mammary epithelial cells are derived from'British Sannen' goat mammary glands. Cells were obtained from Dr. C. Wilde in this institute. Cells were routinely cultured at 37°C in 10 % foetal calf serum (FCS) DMEM(41965-039 Life Technologies) supplemented with penicillin and streptomycin, and used to express green fluorescent protein (GFP) to test the mammalian expression baculovirus system.

D. Antisera

Anti-rat IGFBP-5 antisera was made in house by immunizing sheep with recombinant rat IGFBP-5 protein. Antiserum was by kindly donated by Dr. David Flint, and was first reported in

Bramani *et al.* (1999). This antiserum was routinely used at a dilution of 1:2000 for Western immuno blotting.

Anti-rat IGFBP-2 antisera raised in rabbit was purchased from TCS Bilogicals (Bucks, UK), and used at a dilution 1:5000 for Western blot.

Antisera to sheep or rabbit Imunoglobulin (IgG) conjugated to horse radish peroxidase (HRP) were purchased from Sigma and used at a dilution 1:10000 and 1:20000, respectively.

II.1.4 General techniques for DNA sub-cloning

cDNA clones for rat IGFBP-5 and rat IGFBP-2 were kindly provided by Dr Sean Gunette (John Wayne Cancer Research Institute, Los Angeles, USA) cloned into the EcoRI site of pGEM7Zf (Promega). The full-length IGFBP-5 cDNA contains 869 base pairs (bp) of coding sequence and 95 bp of 5' and 3' untranslated sequences, making the excised EcoRI fragment 964 bp in total. The full-length IGFBP-2 cDNA contains 912 bp of coding sequence and 65 bp of 5' and 3' untranslated sequences, making the excised EcoRI fragment 977 bp in total.

Restriction enzyme digestion. Restriction digests were typically performed in excess of enzyme: 20µl reaction volume containing 1µgDNA, 2µl x10buffer (containing 0.5mg/ml BSA), 10units enzyme. Endonuclease enzymes and appropriate 10x buffers were supplied by Boehringer, Promega or New England biolab. Digestion mixes were incubated at the recommended temperature for 1-2h.

Agarose gel electrophoresis. 1% *(w/v) low melting point agarose* (Sigma) was dissolved in 1xTBE buffer and 0.5µg/ml ethidium bromide was added. Gels were electrophoresed in 1X TBE buffer as described above. Low melting point agarose gels were used for DNA inserts, that were to be excised from the gel and extracted with QIAquick Gel extraction kit (QIAGEN, West Sussex, UK) following the instructions described by the manufacture. Electrophoresed DNA was visualised on a Herolab transilluminator (Mididoc, gel documentation analysis system and EASI store software Herolab Molekulare Trenntechnik,)

Ligation. Leinarization of plasmid DNA with a single restriction enzyme was followed by alkaline phosphatase (Promega) treatment to prevent self-ligation (2µg DNA, 2 units enzyme, 37°C

for 30min, inactivated 65°C for 10min). A typical ligation reaction would be performed in 15µl volume containing: 1.5µl X 10 ligation buffer (500mM Tris-HCl (pH7.5), 100mM $MgCl_2$, 100mM DTT, 10mM ATP, 250µg/ml BSA); 100-200ng of linearized, plasmid DNA, 100-200ng of DNA insert with compatible restriction enzyme ends and 15U T_4DNA ligase (0.5µl of high concentration T_4DNA ligase from New England Biolab, Hertfordshire, UK). Reactions were incubated at 16°C overnight.

Transformation. For a typical transformation, aliquots of JM109 competent cells would be thawed on ice for approximately 10min. 100µl of cell suspension would be placed in polypropylene tubes on ice and after the addition of 50ng of plasmid DNA, tubes would be mixed and incubated on ice for 30min. Tubes were incubated at 42°C for 45 seconds and immediately chilled on ice for 2min. After the addition of 900µl LB medium to each tube, cells were incubated at 37°C for 1h with shaking to allow them time to express the antibiotic resistance gene on the plasmid which they have just taken up before being exposed to this antibiotic. 100µl of cell suspension would be plated onto LB-agar plates in the presence or absence of appropriate drug selection and plates incubated at 37°C for 18-24h.

Minipreps were performed by using Ultraclean™ mini plasmid prep kit (MoBio Laboratories, Solana Beach, CA) to obtain purified DNA that could be subsequently used for most molecular biological techniques. The procedure used was that recommended by manufacture.

Maxipreps were performed using QIAGEN tips 500 (QIAGEN, West Sussex, UK) following the method recommended by the manufacturer.

II.2 Site-directed mutagenesis

In order to introduce the mutations necessary for creating our various IGFBP-5 mutants, we carried out site-directed mutagenesis of the rat IGFBP-5 cDNA (pGEM7Zf rat IGFBP-5 was kindly gifted by Dr Sean Guenette, John Wayne Cancer Institute Los Angeles) using the Promega "Altered sites® II" *in vitro* mutagenesis system. This technique exploits the hybridization between the wild type gene and a single stranded oligonucleotide which has a sequence complementary to the wt gene, but which carries an alteration of a few base pairs at the site of mutagenesis. First, the

IGFBP-5 gene was cloned in pALTERex1 plasmid which contains an inactivated ampicillin resistance gene and an active tetracycline resistance gene. In each mutagenesis reaction, 2 or 3 different oligonucleotides were used: i) one oligo to introduce the desired mutation (various IGFBP-5 mutants), ii) one oligo to repair the ampicillin resistance gene, and iii) one oligo to knock out the tetracycline resistance gene. Subsequent rounds of mutagensis would then employ a tetracyline repair oligonucleotide, and an ampicillin knock out oligonucleotide if required.

T_4DNA polymerase was used to synthesis nascent DNA between the oligonucleotides, using plasmid single strand DNA as template, and DNA ligase was used to ligate the newly synthesised DNA fragments. As a result, a wt-mutant hybrid DNA duplex was obtained. In order to avoid the host-directed mismatch repair system which would repair the unmethylated newly synthesised DNA strand, the hybrid duplex was amplified in the *E. coli mutS* strain (ES1301muts). Due to the semi-conservative mode of DNA replication, wt and mutated plasmids would theoretically be amplified at the same ratio. The introduction of ampicillin selection allows an increase in the yield of mutated DNA over the wt.

II.2.1 Construction of G203K, Q209A, and BP550 mutants

IGFBP-5 cloning into pALTER ex1. The rat IGFBP-5 cDNA was excised from the pGEM vector as an EcoRI fragment, separated in an agarose gel and extracted using QIAquick Gel extraction kit. EcoRI digested pALTERex-1 plasmid was separated by the same procedure as mentioned above. pAlter-IGFBP-5 resulting from this ligation was transformed into JM109 cells, and grown on tetracycline plates for 48h at 37°C. Single colonies were picked and grown in LB-tet medium. Mini and maxi preps were prepared (pALTERex1-IGFBP-5 stock solution was present at 436μg/ml in TE buffer)

Mutagenetic primers. For the construction of G203K, Q209A, and BP550, which has a premature stop codon between the junction of exon 2 and exon 3 of IGFBP-5, mutagenic primers were designed as follows. First, according to the number of nucleotide substituted, the size of the mutagenic primers were adjusted. If one or two nucleotides were substituted, mutagenic primers containing at least 11 or 15 base pairs respectively of flanking sequence at either side of the

mutated nucleotide(s) were designed. This would allow sufficient hybridization to the IGFBP-5 sequence. Second, where possible the mutagenic primers were designed in such a way as to create unique restriction endonuclease sites to make it easy for screening purposes. G203K, Q209A, and BP550 mutagenic primers created unique ApoI, SphI, and ApaI restriction sites, respectively. For G203K and Q209A mutations, the mutagenic primers used were 5'-CCAACTGTGACCGCAAAAATTCA CAAGAGA AAGC-3' and 5'-GATTCTACAAG

ApoI

AGAAAGGCATGCAAGCCTTCTCGTGG-3', respectively. To create the Double mutant, Q209A

SphI

DNA was used as the template and the mutagenic primer used was 5'-CCAACTGTGACCGCAA AAAATTCTACAAGAGAAAGG-3'.

ApoI

To create BP550, the mutagenic primer used was 5'-TGAGACAGGAATCTGACTA GGGCCCCT GCCGCAGACA-3'. All the mutagenic primers were supplied and lyophilised either

ApaI

from Cruachem Ltd (Glasgow, UK) or MWG biotec (Milton Keynes, UK). Each oligonucleotide was resuspended in H_2O at 1μg/ml.

5'phosphorylation of oligonucleotides. 1μg (1μl) of each oligonucleotide was incubated at 37°C for 30min in the presence of 2.5μl of kinase buffer x10 (Promega, Southampton, UK), 0.5U (0.5μl) T_4 polynucleotide kinase (Promega), 2.5μl of 10mM ATP (in a final volume of 25μl). Kinase was then heat inactivated at 70°C for 10min and the reactions stored at –20°C.

Alkaline denaturation reaction. 6μl of pALTER ex1-IGFBP-5 template (2.5μgDNA) was incubated for 30min at 37°C with 1μl of 4M NaOH and 1μl of 2mM EDTA (in a final volume of 20μl). 2μl of 3M Na acetate (pH4.8) was added and DNA was precipitated at –70°C for 30min with the addition of 75μl of 100% ethanol. After centrifugation at 13000rpm for 15min and washing the pellet with 200μl of prechilled 70% ethanol, samples were recentrifuged at 13000rpm for 15min. The supernatants were discarded, pellets were vacuum dried and resuspended in 100μl

TE. DNA denaturation was confirmed on a 1% (w/v) agarose gel, where single stranded DNA runs as a separate lower band.

Annealing reaction. 10µl of alkaline denatured ds DNA template, 1µl Amp-repair Oligo, 1µl Tet-knockout Oligo, 1µl of phosphorylated mutagenic primers diluted 1:3, 2µl of manufacturer's x10 annealing buffer and H_2O to a final volume of 20µl, were incubated for 5min in a 75°C water bath. The water bath was then switched off until the temperature reached 45°C (approximately 30min) and finally tubes were put on ice until room temperature (RT) was reached. The following were added on ice (in this order): 3µl synthesis x10 buffer, 1µl (5-10units) T_4 DNA polymerase, 1µl (1-3units) T_4 DNA ligase and finally H_2O to 30 µl. Tubes were then incubated for 90min at 37°C.

Transformation of ES1301 mutant competent cells. For transformation 1.5µl of mutagenesis reaction (approximately 10ng of template DNA) was used and the general protocol described earlier (page 71) was followed, except that after the addition of DNA, the cells were incubated on ice for only 10min. The cells were then incubated at 37°C for 30min with shaking, and 500µl of each culture was then transferred to a fresh tube containing 4.5ml LB-Amp medium and grown overnight at 37°C with shaking.

Plasmid mini prep. Miniprep preparations, of DNA were undertaken following the protocol described above.

Transforming mutant DNAs into competent cells. The yield of mutated DNAs obtained from ES1301muts by the mini prep preparations was estimated on a 1% *(w/v)* agarose gel. 5-10ng of DNA was transformed into JM109 competent cells. However, the BP550 mutant was transformed into JM110, which does not contain *dcm (DNA cytosine methylation)* methylase. This is because the created restriction endonuclease site in BP550, ApaI, is dcm sensitive. The cells were then grown overnight on LB-Amp plates at 37°C. Mini-preps and maxi-preps were prepared and the presence of mutant DNA was verified by restriction digestion with the diagnostic restriction enzymes. The resulting fragments were separated by electrophoresis on a 1% *(w/v)* agarose gel and the result is shown in chapter III (page 98).

Sequencing analysis of mutant DNA was carried out to verify that the correct mutations were present. 7-10µg of DNA was sent to MWG biotec (Milton Keynes, UK) for automated

sequencing. Either T7 or SP6 primers were used for sequencing, as they flank the multiple cloning site in pAlter-ex1.

II.2.2 Construction of Hep- and Double

For the Hep- (R201L, K202E, K206Q, R214A) and Double (G203K, Q209A) mutations, two rounds of mutagenesis were performed. For the Hep- mutation, R201L, K202E, and K206Q mutations were initially created with the mutagenic primer, 5'-CCTGCCCAACTGTGACCTCGA

XhoI

GGGATTCTACCAGAGAAAGCAGTGCAAG-3', which created unique XhoI restriction site. Using this modified cDNA as a template, a second round of mutagenesis was carried out to mutate R214A with the mutagenic primer, 5'-AGCAGTGCAAGCCTTCTGCAGGCCGCAAACGTGG

PstI

CAT-3', which created a unique PstI restriction site. For the Double mutations, the Q209A mutant cDNA was used as a template DNA. The mutagenic primer used was 5'-CCAACTGTGACCGCAA AAAATTCT ACAAGAGA AAGG-3', which created a unique ApoI site.

ApoI

II.2.3 Construction of BP255, BP225, BP522, and BP552

To construct the chimeric mutant IGFBPs, unique XhoI restriction endonuclease sites were inserted at the junction of exons 1 and 2 in the full-length cDNAs for IGFBP-2 and –5 using the mutagenic primers 5'-TCCGAGTGGTCATCCTCGAGGTCTGCAACCTGCTGTG–3' and 5'-TC

XhoI

CCGAGAGTCTCTCTCGAGCTTGGTTTGCTCGCCGT-3' for IGFBP-2 and –5 respectively.

XhoI

XhoI sites were also introduced at the junction between exons 2 and 3 using the mutagenic primers 5'-TGGCAAGGGGTCCTGGCCTCGAGTGGGCGCAGCTTCTTGG-3' and 5'-TGTCTGCGGC

XhoI

AGGGGCCCTCGAGAGATTCCTGTCTCATCT-3' for IGFBP-2 and –5 respectively. The N- and

XhoI

C-terminal domains were then "cut and pasted" by digesting with XhoI and ligating to create BP255, BP225, BP522 and BP552 chimeras. A further round of mutagenesis was required to remove the XhoI sites and restore the wild type sequence at the junction between IGFBP-2 and –5 exons in the chimeric cDNAs, using mutagenic primers 5'-TCCCGAGAGTCTCTCTCACTGTCT GCAACCTGCTGTG-3' for BP255, 5'-TGTCTGCGGCAGGGGCCAGGAGGTGGGCGCAGCT TCTTG-3' for BP225, 5'-TCCG AGTGGTCATCCTCTATCTTGGTTTGCTCGCCGT-3' for BP522, and 5'-TGGCAAGGGGTCCTG GCTTGGTCAGATTCCTGTCTCATCT-3' for BP552.

II.2.4. Construction of Hep-2, Hep-3, and BP550Hep-

In order to disrupt the heparin binding sites in the central domain of IGFBPs, R136A and R137A mutations were created using WTIGFBP-5, Hep- and BP550 as templates. The oligonucleotide used was 5'-GGCTGTGAAGAAGGATGCAGCAAAGAAGCTGACCCAGTC-3'.

TseI

This oligonucleotide introduced an additional TseI site.

II.3 Protein expression using Baculovirus expression system

Both wild type (wt) IGFBP-2 and –5, and all other mutant proteins were expressed with the Bac-to-Bac™ baculovirus expression system (Life Technologies). In this system the gene to be expressed is cloned into baculovirus and the recombinant virus is used to infect insect cells which express the recombinant protein.

II.3.1 Overview of the system (Figure II.1)

Wt and all the mutant genes were cloned into the donor plasmid, pFastBac, down stream of the polyhedrin promoter of AcNPV (Autographica californica nuclear polyhedrosis virus) baculovirus. In nature, the polyhedrin promoter is activated in the very late phase of baculovirus infection of insect cells. In this phase virus DNA particles are packaged in polyhedrin protein and

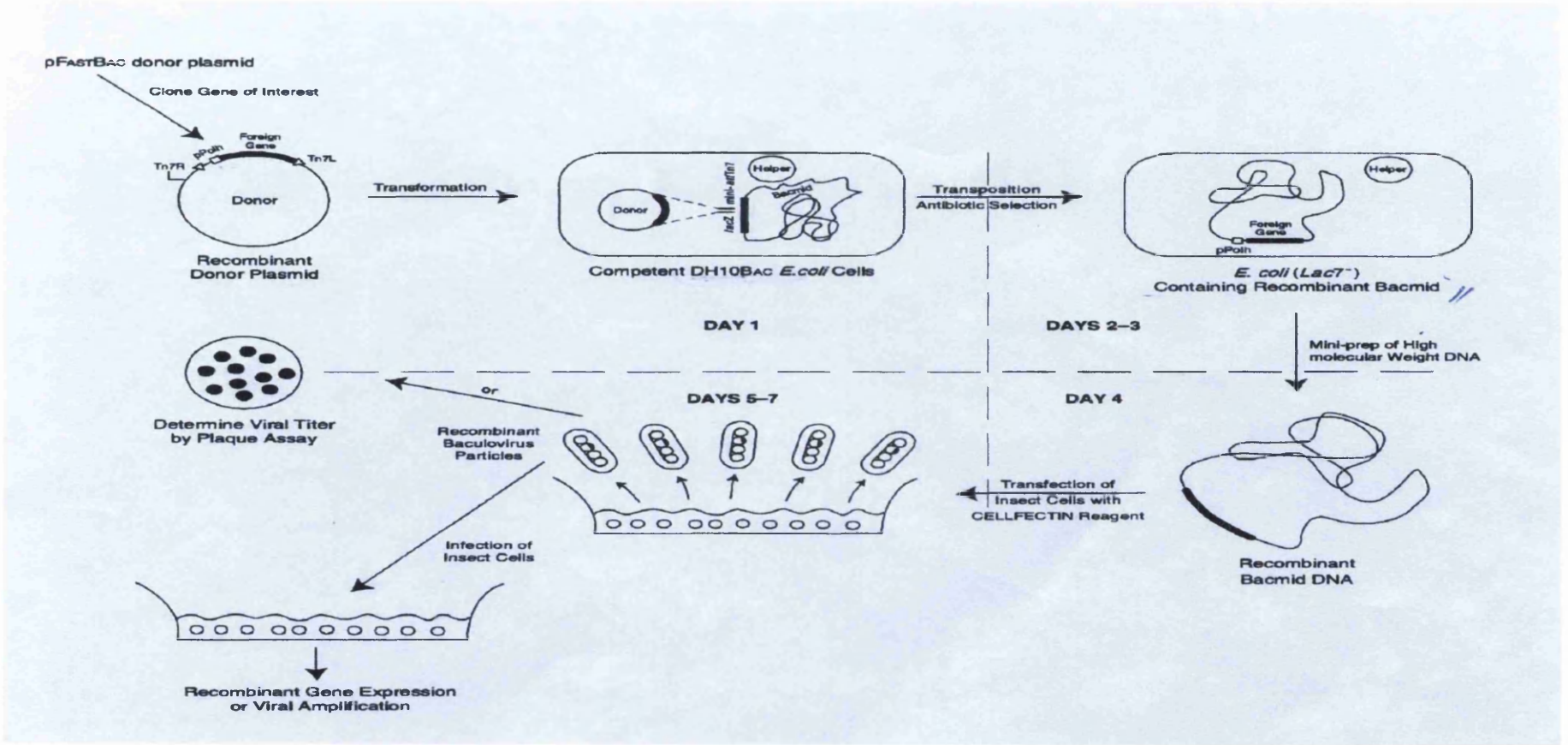


Figure II.1 **Generation of recombinant baculoviruses and gene expression with the BAC-TO-BAC Expression System**
The gene of interest is cloned into a pFASTBAC donor plasmid, and the recombinant plasmid is transformed into DH10Bac competent cells which contain the bacmid with a mini-*att*Tn7 target site and the helper plasmid. The mini-Tn7 element on the pFASTBAC donor plasmid can transpose to the mini-*att*Tn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmin. Colonies containing recombinant bacmids are identified by disruption of the *lacZα* gene. High molecular weight mini-prep DNA is prepared from selected *E-Coli* colonies containing the recombinant bacmid, and this DNA is then used to transfect insect cells.

stored as occlusion bodies. However, the expression of polyhedrin protein is not essential under *in vitro* conditions where the virus can replicate and infect neighbouring cells even if the polyhedrin gene is substituted with a heterologous gene. In pFastBac donor plasmid, the polyhedrin promoter and the gene of interest are located in an expression cassette flanked by the right and left arm of bacterial transposon gene, Tn7.

When pFastBac is transformed into *E. coli* DH10Bac cells, a helper plasmid (pMON7124) provides the functions necessary to transpose the gene of interest from pFastBac to a baculovirus shuttle vector, (the bacmid, pMON14272), contained in the cells. Routinely bacmid can be propagated in *E.coli* DH10Bac cells as a large, low copy number plasmid which confers on the cells kanamycin resistance and the ability to ferment lactose. The bacmid possesses a gene encoding *lacZ*α peptide which compensates the for *lacZ* deletion in the bacterial chromosome. Therefore, on BluoGal (5-bromo-3-indolyl-β-$_D$-galactopyranoside)-IPTG plates, DH10Bac colonies appear blue. Moreover the bacmid has inserted inside the lacZα gene, a mini-*att*Tn7attachment site for Tn7 bacterial transposon. Inside DH10Bac cells the mini-Tn7 element of pFastBac (including the gene of interest and the protein expression cassette) is transposed at the bacmid mini-*att*Tn7 site. The insertion of the mini-Tn7 element into the bacmid causes the disruption of the reading frame of lacZα peptide. Therefore on BluoGal-IPTG plates, colonies containing recombinant bacmid will appear white, allowing an easy selection from the background of blue colonies containing non recombinant bacmid.

Finally, recombinant bacmid is purified and used to transfect a monolayer of Sf9 insect cells. Viral stock is harvested from the supernatant of transfected cells and is utilised to infect fresh insect cells in order to obtain the expression of the protein of interest.

Cloning WTIGFBP-5 and -2, and other mutants into pFastBac1. Both WTIGFBPs and all the other mutants inserts were obtained by digesting approximately 20μg of pALTER recombinant vectors with 4μl (=40units) of appropriate restriction enzymes at 37°C for 2h in a final volume of 60μl with appropriate 1x diluted buffer. DNA inserts were separated in a 1% *(w/v)* agarose gel and extracted with the QIAquick Gel extraction kit.

Analogously, 20μg of pFastBac plasmid were prepared for the ligation reaction by digestion with appropriate restriction enzymes with separation and purification by 1% *(w/v)* agarose

gel loading and QIAquick Gel extraction kit respectively. Relative amounts of pFastBac, WTIGFBPs and all the other mutant DNAs were estimated on a 1% agarose gel by comparison with the intensity of known amounts of DNA in size marker bands. Approximately, 2μg of pFastBac1 were ligated to 2μg of either wt or mutated IGFBP-5 fragments following the general protocol described above. Ligation products were transformed into JM109 cells under ampicillin (100μg/ml) selection. Minipreps of DNA were prepared and a restriction enzyme digestion was performed to detect the recombinant clones. Orientation of the recombinant inserts was verified by multi-restriction enzyme digest. Maxi prep DNA was then prepared for each one of the mutants or wt IGFBP-5 proteins.

Transposition. DH10Bac cells were transformed with 1ng of wt IGFBPs and all the other mutant genes. The general protocol was followed, with the exception that after transformation 900μl of LB medium was added and that the cells were incubated for 4 h at 37°C with shaking to allow them time to express the antibiotic resistance gene on the plasmid which they have just taken up before being exposed to these antibiotics. Each culture of transformed cells was serially diluted with LB (10^{-1}, 10^{-2}, 10^{-3}) and 100μl of each dilution was plated onto appropriate LB agar plates to select for recombinant bacmid DNA (Chapter II; page 64). After 24h incubation at 37°C, 10 large white colonies within a background of small blue colonies were picked, and to confirm the white phenotype, they were re-plated on fresh plates.

High Mr plasmid (Bacmid) mini preps. From each of the wt IGFBPs and all the other mutants DH10Bac plates a single colony with confirmed white phenotype was picked and cultured at 37°C for 24 h in 2ml of LB medium supplemented with kanamycin, gentamycin and tetracycline (at the same concentration reported above for the plates). High Mr bacmid mini preps were prepared by Ultraclean™ mini plasmid prep kit.

Transfection of Sf9 cells with recombinant Bacmid DNA. Sf9 insect cells (>97% viable) were grown in suspension to mid log phase. Cells were then resuspended at $4.5x10^5$ cells/ml in SF900II medium containing 50U/ml penicillin and 50μg/ml streptomycin. 2ml of cell suspension was placed in each well of a 6well plate and cells were left to attach for 1hr at room temperature. In a tube, 100μl of SF900II medium without antibiotics was mixed with 5μl of each recombinant Bacmid. At the same time 100μl of SF900II medium without antibiotics was mixed with 6μl of

Celfectin (Life Technologies) and then added to recombinant bacmid and incubated at ROOM TEMPERATURE for 25min. Wells containing cell monolayers were washed with 2 ml of SF900II without antibiotics. Transfection was performed in the following order: i) 0.8ml of SF900 II was added to each tube containing 200µl of lipid-DNA complexes and the contents mixed. ii) Following complete removal of wash medium from one well, the cell monolayer was overlaid with 1ml of transfection mixture and incubated for 5h at 27°C. The transfection mixture was then removed and 2ml of SF900II medium with antibiotics was added, and the plate was incubated for 72h at 27°C. After this incubation period, the supernatants containing recombinant viruses were harvested. Supernatants from 2 wells were pooled, centrifuged at 1000rpm for 5min, transferred to a 15ml tube wrapped with foil and stored at 4°C. 100µl of this sample was assayed for protein expression by Western blot.

Virus amplification . In order to obtain a large volume of high titre viral suspension necessary to innoculate 50-100ml of insect cell cultures, the supernatants harvested after 48h from insect cells transfected with recombinant bacmids underwent several rounds of amplification. Insect cells were seeded at confluent density on 6 well plates or flasks. The viral innoculum was diluted in a volume of SF900II just sufficient to cover the cell monolayer, which was then incubated for 1h at room temperature. The innoculum was discarded and replaced with fresh SF900II medium, and cells were incubated for 48hr at 27°C. Supernatants were harvested, centrifuged at 1000rpm for 5min to eliminate residual cells, transferred to bottles wrapped with foil and stored at 4°C.

Virus titration Titration of virus was performed with the BacPAK™ baculovirus rapid titer kit (Clontech, CA USA, supplied by Cambridge Bioscience, Cambridge, UK) following procedures recommended by the manufacturer.

Protein harvesting For protein production, a 50ml suspension culture of SF900II medium containing 2 x10^6 cells/ml of Sf9 cells in log phase was infected with a variable volume of virus innoculum. Virus infection was performed at a multiplicity of infection (MOI) of 5 using the following formula:

$$\text{innoculum required (ml)}: \quad \frac{\text{desired MOI(pfu/ml)} \times \text{(total number of cells)}}{\text{titre of viral innoculum (pfu/ml)}}$$

During the infection, samples were taken at 8hr intervals to check the cell viability and cultures were harvested when the viability dropped below 80%. The samples were also analysed on Western blots for the expression and possible degradation of the protein, in an attempt to optimise the harvesting time.

Insect cells were separated from the media by centrifugation at 3000rpm for 2min, supernatants were concentrated on Millipore tubes (Ultrafree-20) by centrifugation at 1700xg for 2hr at 4°C, and stored at -20°C.

II.4 IGF-II affinity chromatography purification

II.4.1 Affinity column preparation

Columns were prepared and run following the recommendations described in "Affinity chromatography. Principle and methods" hand book (Pharmacia LKB biotechonology Cat.N 18-1022-29). 500ug of activated CM Sepharose 4B (Cat N. 17-0490—01 Pharmacia Biotech, Uppsala Sweden) was washed in a sintered glass filter with 150ml of 1mM HCl. 0.9mg of rhIGF-II (media grade GroPep) was dissolved in coupling buffer (0.1M $NaHCO_3$, pH 8.0) and mixed with the gel end over end for 1hr at room temperature. Sepharose gel coupled with IGF-II was poured into a Biorad plastic column and, after discarding the excess coupling buffer, was washed first with 100ml of 0.05M Tris-HCl, 0.5M NaCl pH 8.0, then with 100 ml of 0.05M formate, 0.5M NaCl, pH 4.0. Excess activated CM groups were blocked with 0.1M Tris-HCl pH 8.0 and the column re-equilibrated in 50mM Tris-HCl, 0.5M NaCl, 0.01% sodium azide pH 7.4. The column volume was 1ml.

II.4.2 Purification of wt IGFBPs and mutant proteins and Iodination of IGFs

Protein purification was performed following the method described by Carr *et al.* (1994). 800µl IGF-II-coupled Sepharose gel, prepared as described above, was equilibrated with 10ml equilibration buffer (0.5M NaCl, 50mM Tris-HCl pH 6.5). 2- 4ml of concentrated insect cell culture

supernatant containing wt IGFBPs and mutant proteins were applied to the column. The gel was mixed gently with the sample several times and incubated overnight at 4°C. Unbound protein solution was run through the column, reapplied twice and finally harvested and kept at –20°C to be analysed for the residual presence of IGFBPs. The column was washed with 10 ml equilibration buffer (0.5M NaCl, 50mM Tris-HCl pH 6.5) (the first 1ml of the flow through was retained for analysis). 6 ml of elution buffer (0.5M acetic acid pH3) was incubated with column gel for 10min before starting the elution. 6x 1ml fractions were collected in Eppendorf tubes already containing 275µl 2M Tris base necessary for immediate pH buffering to pH 7.0. Fractions were kept on ice and the column washed with 10ml equilibrium buffer (0.5NaCl, 50mM Tris-HCl pH 6.5). Subsequently, alternate washes with 5ml of basic washing buffer (0.1M Tris-HCl, 0.5 NaCl, pH 8.5) and 5 ml of acidic washing buffer (0.1M Na acetate, 0.5M NaCl, pH 4.5) were repeated twice. For storage the column was equilibrated in column storage buffer (50mM Tris-HCl, 0.5 M NaCl, 0.01% *(w/v)* sodium azide, pH 7.4) and kept at 4°C.

WTIGFBPs and all the other mutants fractions eluted from the affinity column were analysed for their relative protein content by Coomassie blue gels and the presence of purified proteins was confirmed by anti IGFBP-5 and –2 antisera. Western immunoblots and ^{125}I-IGF-I/-II ligand blots. Measurement of protein concentration was by Bradford assay (see below).

Iodination of IGFs. IGF-I and –II were iodinated with Na^{125}I (ICN, Hampshire, UK) to a specific radioactivity of 130 MBq/umol by the iodogen-coated tube method (Fraker and Speck, 1978)

II.5 Protein analysis using Bradford assay

Proteins were quantified using the Bio-Rad protein assay system (Pierce, Chester, UK) which is adapted from Bardford *et al.* 1976. Five dilutions of a BSA standard in elution buffer to pH 7.0 with concentrated Tris buffer (page 65), 2M Tris (pH 7.0), which was an appropriate blank for the protein solutions to be tested were prepared as a standard solution. The linear range of the assay was 5.0 µg/ml to approximately 100 µg /ml. Standard and protein sample solutions were normally assayed in triplicate. 160 µl of each standard and sample solution were pipetted into

separate microtitre plate wells, and then 40 µl of diluted dye reagent concentrate was added to each well and mixed thoroughly using a multi-channel pipette. Samples were incubated for 10min at room temperature, and loaded into a TitreTeck® multi-scan and absorbance measured at 600nm.

II.6 Immobilized protein assay

II.6. 1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out following the method described by Laemmli (1970). Protein gels contained 12.5% or 16% total acrylamide (page 67). Polymerisation was initiated with ammonium persulphate (Biorad) and TEMED. Stacking gels were as described on page 67. Samples for analysis were mixed 1:1 with sample buffer (page 65). Electrophoresis was performed at constant voltage (150V) on a Biorad mini protean II apparatus in runnig buffer (page 65).

Coomassie blue staining. This technique was employed to visualise protein bands after electrophoretic separation and to estimate their relative concentration. Gels were stained in 5% (v/v) methanol, 7.5% (v/v) acetic acid, 0.3% (w/v) Comassie blue R250 by shaking gently for approximately 1hr at room temperature. To enable the detection of protein bands from the stained background, gels were washed for 10min in 5% (v/v) methanol, 7.5% (v/v) acetic acid, then fresh solution was replaced and gels destained overnight. For further analysis, gels were dried between cellophane sheets with Hoefer Easy breeze gel dryer equipment. Alternatively, if direct autoradiograhy was to follow, gels were placed on filter paper (Biorad), covered by Saran film and dried on a Hoefer (Hoefer scientific instruments, S.Francisco, CA) dual temperature slab gel drier at 80°C for 2h and then at room temperature until completion.

Electrotransfer. As an alternative to direct gel staining, proteins separated by electrophoresis were transferred from the gel to either Hybond-C extra or Hybond-PVDF (Amersham) membranes. In the latter case membranes required 10sec pre-wetting in methanol and subsequent 5min rinsing in H_2O before use. Gel sandwiches (fibre pad, filter paper, gel, membrane, filter paper, fibre pad) were assembled under transfer buffer (25mM Tris, 192mM glycine, 20% (v/v) methanol) in a bath and then located in the Biorad mini transblot apparatus equipped with an ice pack. Protein electrotransfer was performed at a fixed voltage (100V) for 1h, unless otherwise

indicated. Blotted proteins were usually visualised by Ponceau S staining, as described below. Membranes were then either blocked for ligand or Western blotting for immediate analysis or dried and stored between filter paper in a plastic bag at 4°C, for later analysis. Dried PVDF membranes were moistened for 10sec in methanol before being further processed.

Ponceau S staining. Sigma Ponceau S concentrated solution (Ponceau S, 2% *w/v*, in TCA, 30% *w/v*, and sulphosalicylic acid, 30% *w/v*), diluted 1:10 with water, was applied to the membranes for 10min for visualisation of protein bands. After washing with water, the membranes were dried and stored or directly incubated in blocking buffer for further analysis by ligand or Western blotting.

II.6.2 ^{125}I-IGF-I/-II ligand blot

^{125}I-IGF-I/-II ligand blotting was performed essentially as described by Hossenlopp *et al.* (1986). After transfer, membranes were washed with 50ml 3% (v/v) NP40 Tris-saline solution (10mM Tris-HCl, 60mM NaCl 0.05% (w/v) sodium azide, pH 7.6) for 30min, with 50 ml 1% (w/v) BSA Tris-saline solution for 2h and finally with 50ml 0.1% (v/v) Tween 20 Tris-saline solution for 10min. Approximately 1.5-2 x 10^6cpm ^{125}I-IGF-I/-II in 1.5ml of 1% (w/v) BSA, 0.1% (v/v) Tween 20 Tris-saline solution was added to a plastic bag containing each blot and incubation was continued overnight at 4°C. Blots were then washed twice for 15min in 50ml 0.1% (v/v) Tween20 Tris-saline solution and three times for 15min in 50ml Tris-saline solution. After drying, membranes were exposed to autorad films (Hyperfilm βmax, Amersham) in an 18x24 radiographic cassette (Genetic Research Instrumentation Ltd. Essex, UK) at –70°C for 4-10 days. The films were bathed in GBX Kodak developer for approximately 3min followed by a quick rinse in water, moved to fixer solution for 5min and then extensively washed in tap water.

As an alternative to autoradiographs, dried blots were exposed to a Molecular Dynamics Phospho-imager screen for 2 days at room temperature and the resulting image was visualized with the PhosphoImager 445SI software (Molecular Dynamics) and Image Quant™ software.

II.6.3 Western immunoblot

Typically, blotted membranes were blocked in 3% BSA TBS-T (20mMTris- HCl, 137mM NaCl, pH 7.6 supplemented with 0.1% v/v Tween) for 1hr at room temperature or overnight at 4°C. After rinsing in TBS-T, membranes were incubated with the first antibody for 1hr at room temperature in a plastic bag containing approximately 1.5ml of 1-3% (w/v) BSA TBS-T. The dilution and the exact nature of first antibody varied as specified in each experiment. Blots were washed x2 quickly, x1 for 15min and x2 for 5min with TBS-T, with shaking. HRP-conjugated second antibody, was added to 50ml of 5% (w/v) skimmed dried milk TBS-T in which each membrane was incubated for 1h at room temperature, with gentle agitation. After washing in TBS-T, as described above, protein detection would be performed with Enhanced Chemi-Luminescence technique (ECL™ Amersham, Buckinghamshire, UK) by exposing blots for various lengths of time to Hyperfilm™ ECL™ (Amersham). Finally the films would be processed as described above in developer and fixer.

II.6.4 Heparin ligand blot

The method used was adapted from that described previously by others (Grulich-Henn *et al.* 1998) for detection of serum IGFBPs by blotting with biotinylated IGF-I. 200ng of each protein was subjected to electrophoresis on 12.5% or 16% SDS-PAGE under non-reducing conditions and subsequently transferred onto Hybond™-C Extra membranes (Amersham). After transfer, the filters were dried at 37°C for 5min. The membranes were then washed with 3% (v/v) NP_{40} Tris-saline (10mM Tris-HCl, 0.15M NaCl pH 7.4) for 30min and blocked with 3% (w/v) BSA- Tris-saline for 2hrs at room temperature with shaking. They were then incubated overnight with a 1:2000 dilution of Heparin-Albumin-Biotin (Sigma, USA) in 1% (w/v) BSA/0.1% (v/v) Tween-20- Tris-saline at room temperature. The membranes were washed (3 x 15min) with 1% (w/v) BSA/0.1% (v/v) Tween-20- Tris-saline and then incubated with a 1:2,000 dilution of streptavidin-HRP conjugate (Boehringer Mannheim) in 1% (w/v) BSA/0.1% (v/v) Tween-20- Tris-saline for 1hr. Finally, the membranes were washed (3 x 15min) with Tris-saline, and bound heparin was detected using the Enhanced Chemi-Luminescence technique (ECL™ Amersham, Buckinghamshire, UK) by

exposing blots for various lengths of time to Hyperfilm™ ECL™ (Amersham). Protein loading was monitored by blotting duplicate membranes with antiserum to WTIGFBP-5.

II.7 Solution phase assay

Competition studies were established to determine the affinity of wt IGFBP-2/-5, and the other mutants for IGF-I or IGF-II. Both wild type and mutant preparations were diluted 1:2500 with assay buffer and 100µl were added to radio immuno assay (RIA) tubes. 100µl of assay buffer +/- increasing amount of cold IGF-I or IGF-II were added. 1mg/ml IGF-I and II stock solutions in 10mM acetic acid were appropriately diluted to obtain a final concentration of 0.25, 0.5, 1, 2.5, 5, 10, 25, 50ng/ml. Subsequently, approximately 25000cpm of ^{125}I-IGF-I or IGF-II in 100µl of assay buffer were added so that wild type proteins and mutants were each tested for all combinations of competitive binding (IGF-I v ^{125}I IGF-I and IGF-II v ^{125}I IGF-II). After overnight incubation at 4°C, 600µl of 1% (w/v) activated charcoal solution containing 0.2% (w/v) protamine sulfate was added and centrifuged (3000rpm X 30min) to separate unbound tracer. Blank value for each of ^{125}I-IGF-I and ^{125}I-IGF-II tracers was determined and the radioactivity measured in all other charcoal pellets was subtracted from the appropriate blank as described above. ^{125} I IGF-I/II binding of wt or mutated IGFBP-5 proteins in the absence of cold IGFs was designated the control value. The residual tracer binding in the presence of cold IGF competitors was expressed as either a percentage of control value (100%) or a linear regression curve by Scatchard analysis (Scatchard, 1949)

II.8 BIACORE analysis

Kinetics of wt IGFBPs and mutants binding to both IGF-I and IGF-II was investigated using a BIACore 2000 system (BiaCore, Herts, UK).

BIACORE 2000 system is for real-time biomolecular interaction analysis (BIA) using surface plasmon resonance (SPR) technology. This system monitors the formation and dissociation of biomolecular complexes on a sensor surface as the interaction occurs. By covalently attaching one molecule (the ligand) to the surface, the binding of another molecule in solution (the analyte) with the ligand is followed. Measurements are made under condition of continuous flow, where the

sensor surface forms one wall of the flow cell. For the majority of applications, the biospecific surface can be regenerated and reused for an extended series of analyses.

SPR is a non-invasive optical measuring technique which measures the mass concentration of biomolecules in close proximity to a specially prepared surface. The technique does not require any labeling of the interacting components. The response is largely independent of the nature of the biomolecule, so that all steps in an interaction analysis may be followed with the same detection technique.

This system measures surface protein concentrations from a few pg/mm^2, and can monitor changes in the surface concentration on a time scale down to 0.1 second. The technology allows determination of binding specificity, kinetics and concentration with analytes ranging in size from about 150 to 10^6 g/mole (dalton) or more. Flow-through technology with integrated microfluidics gives reproducible sample handling and simplifies kinetic calculations. Finally, this system can be used for studying the interactions of proteins, protein conjugates, nucleic acids, lipid micelles and even larger particles such as viruses and whole cells.

Sensor chip (CM5) is a general purpose chip coated with carboxy-methylated dextran to which biomolecules can be linked with a variety of established chemical methods. CM5 is glass slide coated with a thin gold film to which the carboxy-methylated dextran surface matrix is covalently bonded (Figure II.2). This chip is mounted on a plastic support frame in a protective cassette.

Integrated μ-Fluidic Cartridge (IFC) The IFC consists of a series of channels and pneumatic valves encased in a plastic housing, and serves to control delivery of liquid to the sensor chip surface. Protein samples are injected from the autosampler (automatic sample injection pump) into the IFC, which connects directly with the detector flow cells. The detector flow cells are formed directly on the IFC by docking the sensor chip on to the IFC flow cell block. This consists of four grooves, rectangular in cross section, moulded into the surface of the IFC. Each groove has its own liquid delivery channel and control valve in the IFC. The block is pressed against the sensor chip, forming four separate flow cells with sensor chip surface as one wall. Effluent from the flow cells is collected in an outlet channel and directed either to waste or the recovery cup. The IFC allows single or multichannel analysis in up to four flow cells (Figure II.3). Sample injection

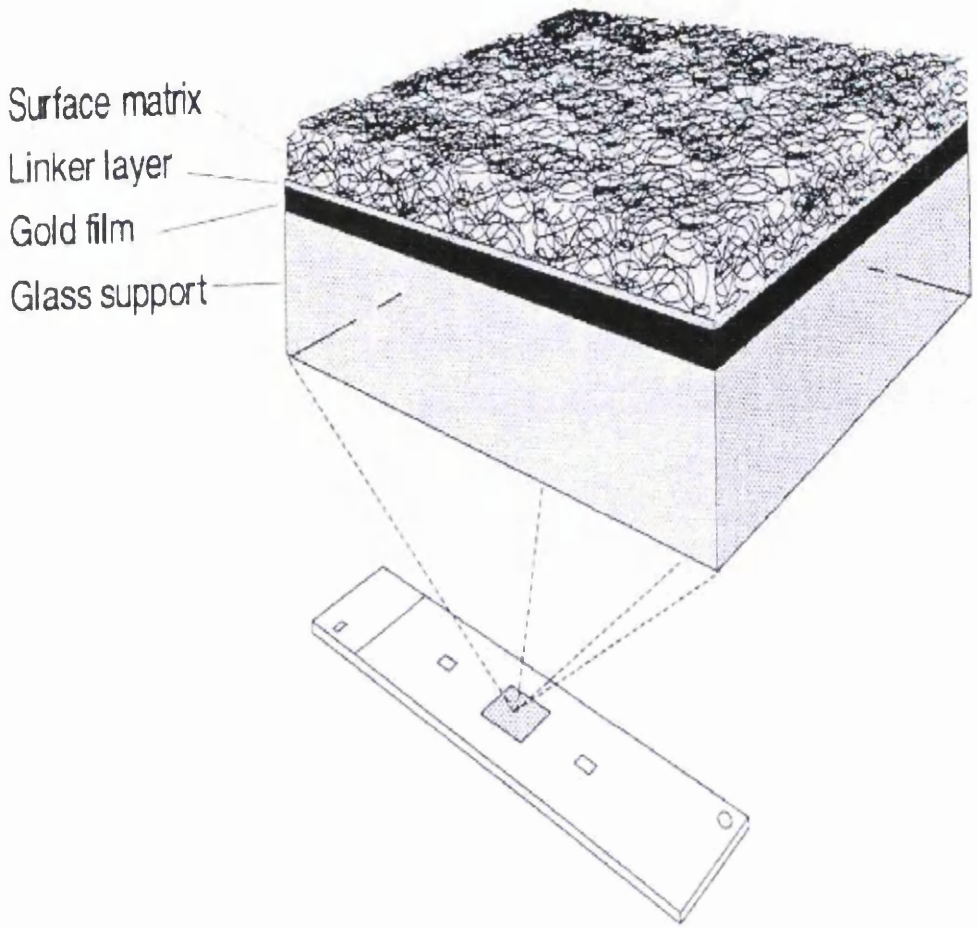


Figure II.2 Sensor Chip CM5 is a glass slide coated with a thin gold film to which the carboxymethyl dextran surface matrix is covalently bonded. The matrix surface forms one wall of the detector flow cell.

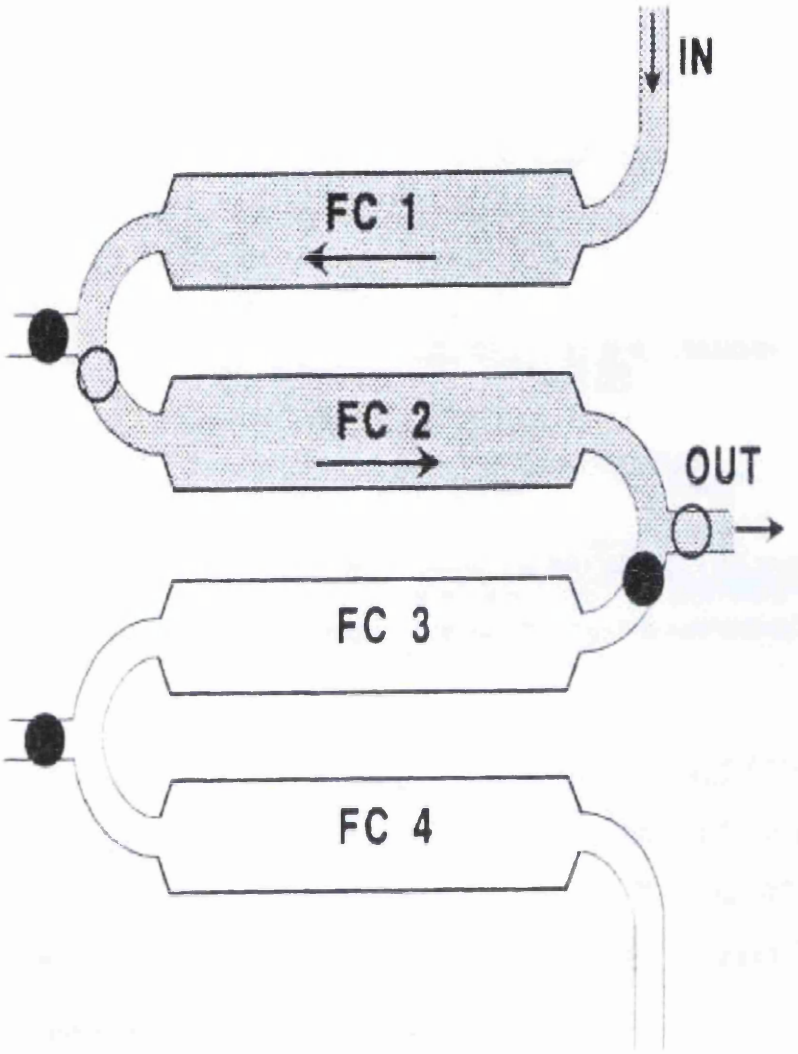


Figure II.3 Simplified diagrammatic illustration of sample flow through two flow cells. The symbols ○ and □ represent open and closed valves respectively.

commands activate a sequence of operations covering aspiration of sample from the autosampler vial, delivering the required amount of sample to the flow cells, and flushing the IFC with buffer ready for the next injection.

Immobilizing IGF-I and IGF-II onto CM5 and protein analysis. IGF-I and IGF-II were immobilized to flow cell number 2 and 3 respectively in the CM5 chip via NHS/EDC, which are coupling reagents to the flow cell surface, according to the manufacturer's instructions. In the control flow cell, insulin (which does not bind IGFBP-5) was similarly immobilized on flow cell number 1. Each polypeptide was immobilized to a level of 1000 reponse units (Rus).

Both wild types and mutant proteins were applied simultaneously to the sensor chip containing IGF-I, IGF-II or insulin at a flow rate of 30 ul/min. Five different concentrations (1.09, 2.18, 4.37, 8.75 and 17.5 nM) of proteins were applied. The regeneration of biosensor chip surface following association and dissociation of binding protein was achieved with sequential 2 x 2 min pulses of 20mM HCl. Under these conditions the biosensor surface was regenerated without any time-dependant increases in background RU signal. Kinetic values (Kon and Koff) were calculated assuming a 1:1 stoichiometry of binding between IGFs and proteins and using BIA evaluation 3.0 software provided by the manufacturer.

II.9 Construction of mammalian cell line expression Baculovirus vector

The baculovirus system is being used increasingly to express proteins in mammalian cells. The receptor for baculovirus on mammalian cells is not known, but it is likely to be a glycosaminoglycan or other ubiquitous cellular component, given the wide range of cell types susceptible to infection. As AcNPV (*Autographa californica* nuclear polyhedrosis virus) is only able to replicate in cells derived from lepidoptera (moths and butterflies), growth of AcNPV in the laboratory usually requires less extensive biosafety precautions than animal viruses. We have constructed a hybrid mammalian-baculovirus expressing vector (Figure VI.1) containing two expression cassettes controlled by constitutive mammalian promoters: the cytomegalovirus immediate early (CMV-IE) promoter and the simian virus 40 (SV40) early promoter controlling neomycin phosphotransferase II. The virus is prepared and amplified using normal insect cell culture procedure (as shown in chapterII.3), although the virus does not contain the insect cell

specific polyhedrin promoter. Using this virus, we have expressed green fluorescence protein (GFP) as a reporter gene in several mammalian cell lines, and both wt IGFBPs and other mutants in mouse C2 myoblast cell lines.

II.9.1 Construction of recombinant Baculoviruses

pBacMam1 was constructed by using shuttle vectors derived from pFastBac1 (Life Technologeis). Plasmid DNA was digested with SnaBI and HpaI to remove the baculovirus polyhedrin gene promoter sequences. A 3.1-kbp NruI-Bst1107I fragment from pcDNA3 (Invitrogen), which contains the cytomegalo virus – immediate early (CMV-IE) promoter/enhancer with a multiple cloning site and polyadenylation signal followed by the simian virus 40 promoter – neomycin phosphotransferase II expression cassette, was inserted into the pFastBac1 backbone.

pBacMam GFP. Green fluorescence protein gene was initially inserted into pBacMam1 as an EcoRI – XbaI fragment to construct pFastBacMam1 GFP reporter gene construct.

pBacMam wt IGFBP-5/Hep-/Double. Wt-IGFBP-5, Hep-, and Double gene were cloned from pFastBac1 to pBacMam1 as BamHI – XhoI fragments.

Recombinant viruses were generated by using the Bac-to-Bac system (Life technologies) as explained above in chapter II.3. Harvested stock viruses were concentrated by centrifugation at 35,000 X g for 60min, and pelleted virus was resuspended in Dulbeco's PBS (Life technology) without serum. Virus titres were determined by the BacPAK^{TM} baculovirus rapid titer kit (Clontech) following procedures recommended by the manufacturer.

II.10 Expression GFP in C2 myoblast cell line and primary goat mammary epithelial cells

Virus infection and expression of GFP. C2 myoblast cells were seeded on gelatin coated 6-well culture dishes (cellstar) at 2 X 10^6 cells/well and incubated for 3 hours at 37°C. Culture medium was removed and cells were washed with three times with 2ml PBS to eliminate traces of serum. After removal of PBS, cells were treated with 1ml volume of virus innoculum (MOI 200 – 400) for 12 –24 hrs at 37°C. 10mM Sodium butyrate was immediately added after virus treatment

to increase the infection rates following the method of Condreay *et al*, 1999. After incubation with virus, the virus innoculum was removed from the cells, and the cells were washed three times with 2ml PBS and trypsinised. Cells were transferred into gelatin coated T75 flasks, and infected cells were then selected with 10 ml of fresh media containing 500ug/ml G-418 (Sigma).

Chapter III. Overlap of IGF- and heparin-binding sites in rat IGFBP-5

III.1 Introduction

As discussed above in chapter I.3.3, several laboratories have begun to identify the specific amino acids in the IGFBP molecule that contribute to IGF binding, and in the study by Forbes *et al.*, (1998) they identified a critical region for binding IGFs in the C-terminus of bovine IGFBP-2 (FigureI.4, Page 26). This critical region of bIGFBP-2 corresponds to amino acids 201-216 in the rat IGFBP-5 (rIGFBP-5) sequence, which lies within the basic heparin binding region of the protein, which we already discussed in chapter I.3.4. We carried out an alignment of sequeneces from all six IGFBPs from all species sequenced so far and found two completely conserved amino acids (Gly 203 and Gln209 in the rIGFBP-5 sequence) (Figure III.1), which, in turn, suggests an important conserved function for these two amino acids. Therefore, we decided to mutate these residues to assess whether they could be involved in IGF binding and/or heparin binding. These two individual mutants of rIGFBP-5 are called G203K and Q209A (Figure III.2). Subsequently, we made a third mutant in which both of these residues were mutated (G203K/Q209A), and we have called this the Double mutant. In addition, in order to assess the overall contribution of the C-terminus of IGFBP-5 to IGF binding, we introduced a premature stop codon at the junction between the central and C-terminal domains of rIGFBP-5 to make a truncated mutant named BP550. Finally, we have made a fifth mutant named Hep-, in which mutations were made in four of the flanking basic amino acids, previously shown by others (Parker *et al*, 1998) to be involved in heparin binding (R201L, K202E, K206Q, and R214A) (Figure III.2).

III.2 Results

III.2.1 Construction of G203K, Q209A, Hep-, Double, and BP550

To create G203K and Q209A, site-directed mutagenesis was performed at two separate sites on the rat IGFBP-5 cDNA using the 'Altered sites' system (Promega) as described in chapter II. The oligonucleotides for creating both G203K and Q209A also generate unique restriction

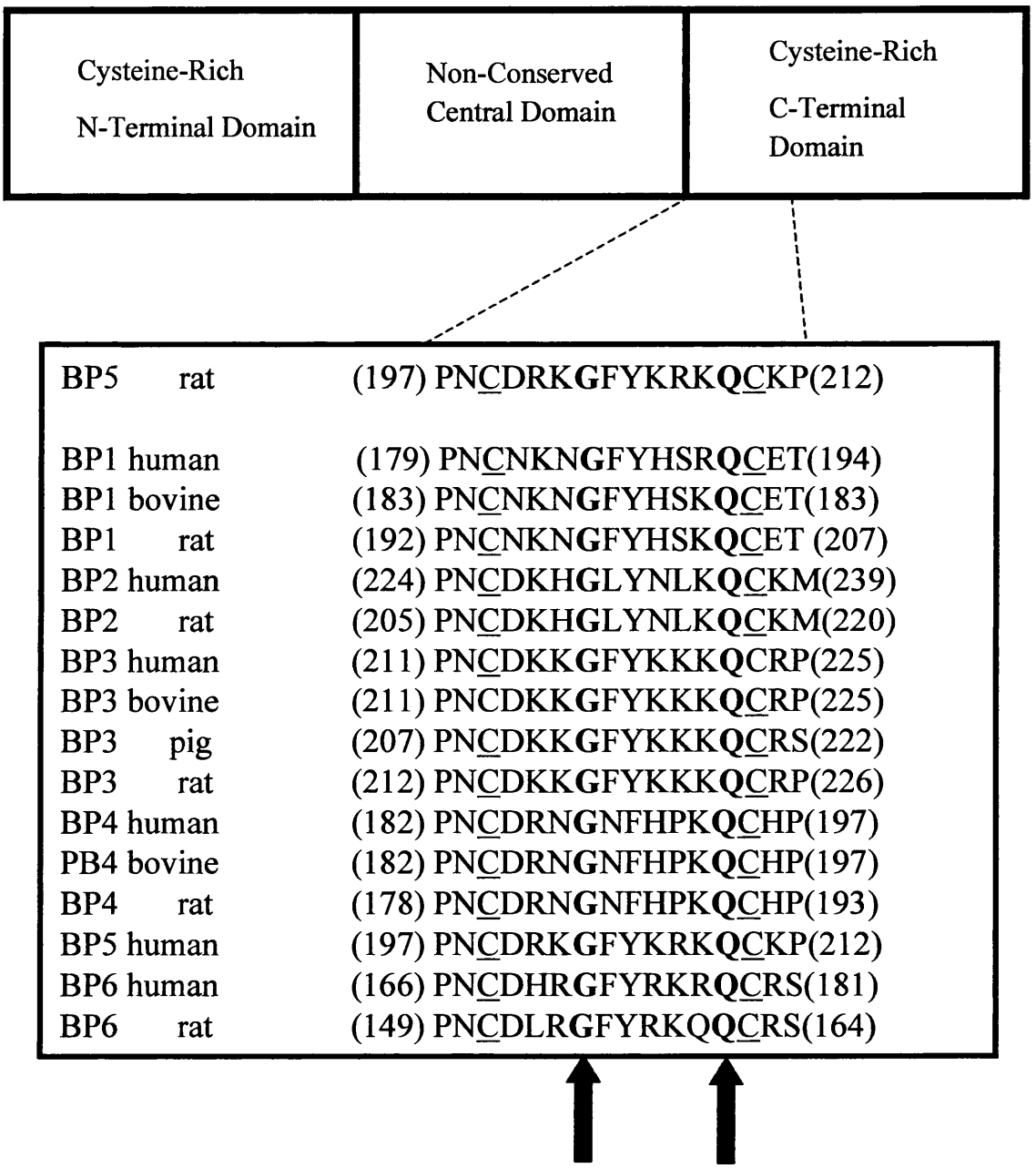


Figure III.1 Alignment of sequences for IGFBPs corresponding to rat IGFBP-5 residues 197-212 The 15 sequences are aligned according to the location of conserved cysteine residues (underlined) at residues 199 and 210 for rat IGFBP-5. This alignment clearly indicates the conservation of residues corresponding to glycine 203 and glutamine 209 in rat IGFBP-5, and these residues are shown in bold and indicated by arrows

G203K

Arg-Lys-**Gly**-Phe-Tyr-Lys-Arg-Lys-Gln-Cys-Lys-Pro-Ser-Arg-Gly-Arg-Lys-Arg

⇓

Lys

Q209A

Arg-Lys-Gly-Phe-Tyr-Lys-Arg-Lys-**Gln**-Cys-Lys-Pro-Ser-Arg-Gly-Arg-Lys-Arg

⇓

Ala

Hep-

Arg-Lys-Gly-Phe-Tyr-**Lys**-Arg-Lys-Gln-Cys-Lys-Pro-Ser-**Arg**-Gly-Arg-Lys-Arg

⇓ ⇓ ⇓ ⇓

Leu Glu **Gln** **Ala**

Double

Arg-Lys-**Gly**-Phe-Tyr-Lys-Arg-Lys-**Gln**-Cys-Lys-Pro-Ser-Arg-Gly-Arg-Lys-Arg

⇓ ⇓

Lys **Ala**

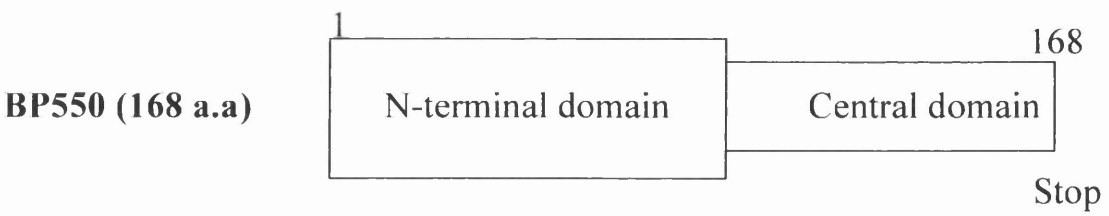


Figure III.2 Site directed mutagenesis of 201-218 region of IGFBP-5 and BP550

endonuclease cleavage sites that are ApoI and SphI, respectively. Figure III.4 shows the results of ApoI and SphI restriction analyses of WTIGFBP-5, G203K, and Q209A cDNAs, inserted in the pFastBac1 plasmid. Although there are no ApoI or SphI sites within the wild type rIGFBP-5 cDNA sequence, there are five ApoI and one SphI restriction sites within the pFastBac1 plasmid (Figure III.3). The BP5 cDNA is cloned into the unique EcoRI site (GAATTC), which also corresponds to an ApoI site (PuAATTPy). Therefore, the expected size of fragments generated from ApoI digestion of pFastBac1WTIGFBP-5 are 3628, 964 (corresponding to the BP5 cDNA), 495, 379, 262 and 11 (undetected) base pairs (bp) (Figure III.4A). After successful mutagenesis to create G203K, the new ApoI restriction site within the IGFBP-5 sequence cuts within the 964 fragment to generate new 721 bp and 243bp fragments, the latter of which is not resolved from the 262 bp pFastBacI fragment (Figure III.4A). pFastBac1WTIGFBP-5 is linearised following digestion with SphI to create a single 5739 bp fragment (Figure III.4B). After successful mutagenesis to create Q209A, the new SphI restriction site within the IGFBP-5 sequence results in the release of a 300 bp fragment (229 bp from the Bp5 sequence and 71 bp from pFastBacI) (Figure III.4B).

To create the Double mutant, Q209A was used as the template DNA for site-directed mutagenesis and the mutagenic primer was that described in chapter II. After mutation, restiction enzyme digestion analysis indicated the prescence of both ApoI and SphI sites (data not shown). The Hep- mutant (R201L, K202E, K206Q, and R214A) was created following two rounds of mutagenesis. Firstly, R201L, K202E, and K206Q were produced, creating a unique XhoI restriction site in the IGFBP-5 cDNA. Using this mutated cDNA as a template, a second round of mutagenesis was performed to create R214A, which introduced a unique PstI site within the IGFBP-5 cDNA. Figure III.5A shows the results of both XhoI and PstI restriction digestion analyses of WTIGFBP-5 and Hep- DNA, inserted in the pFastBac1 plasmid. Although there are no XhoI or PstI sites within the wild type rIGFBP-5 cDNA sequence, there is a single XhoI and PstI restriction site within the polylinker of the pFastBac1 plasmid. Therefore, digestion with either XhoI or PstI of pFastbac1WTIGFBP-5 results in the linearisation of the plasmid (Figure III.5A). However, after succesful mutagenesis to create Hep-, the new XhoI restriction site within the IGFBP-5 sequence results in the release of a 317 bp fragment (252bp from the IGFBP-5 sequence and 65 bp from pFastBac1), whereas the new PstI restriction site results in the release of a 270 bp fragment (212bp

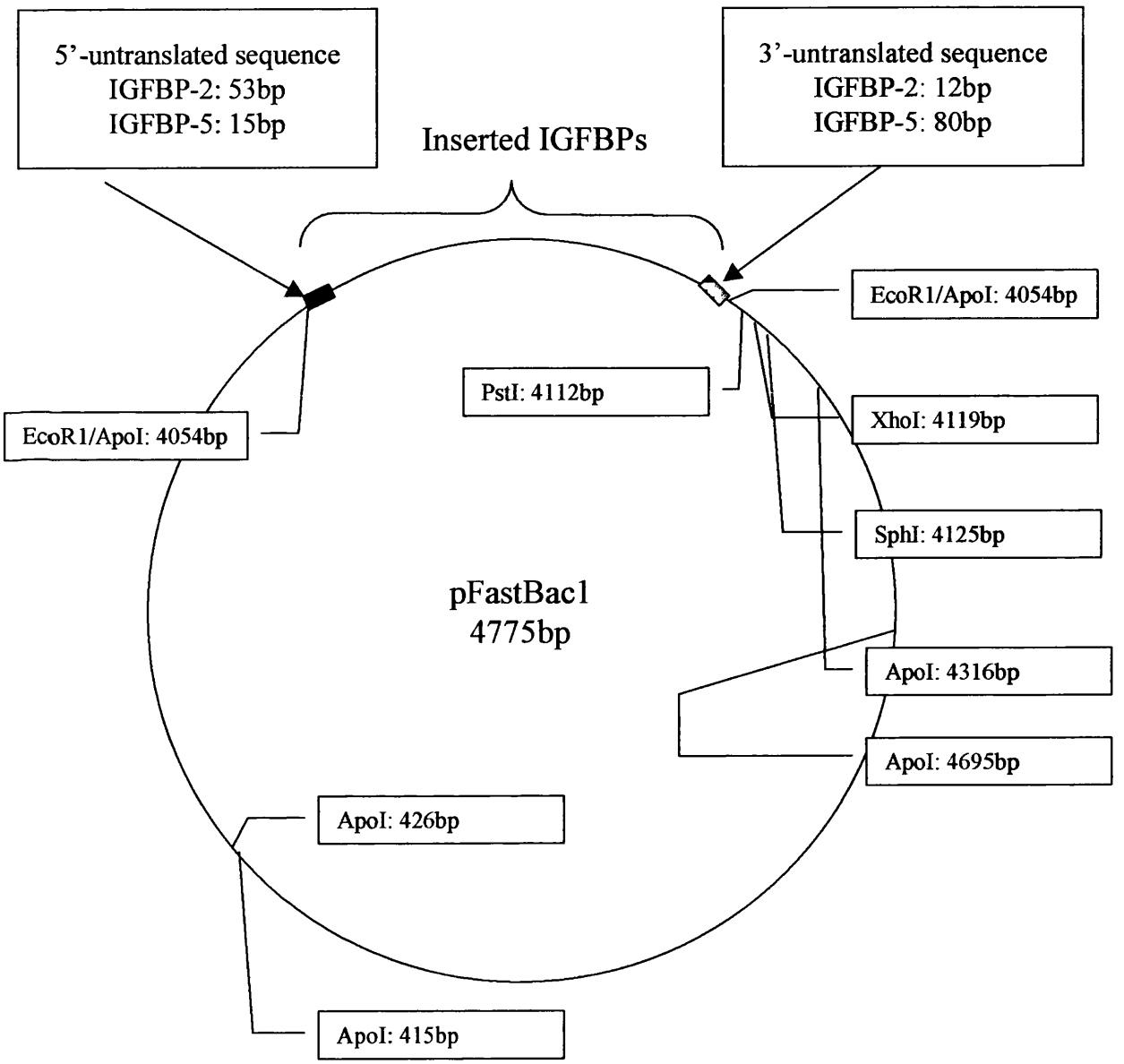


Fig III.3 pFastBac1restriction map. Multiple cloning site contains EcoRI, ApoI, PstI, XhoI, and SphI restriction sites. Four other ApoI restriction sites are found in other region of vector.

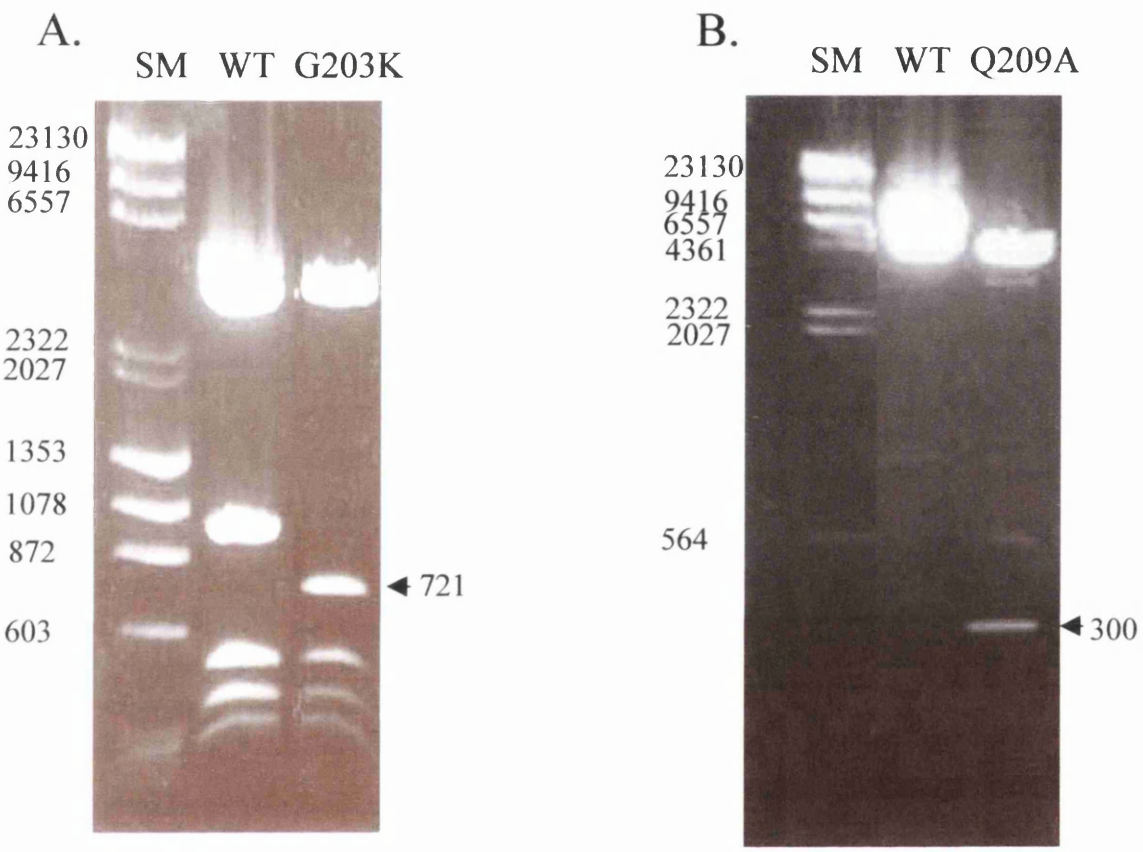


Figure III.4 1% agarose gels stained with ethidium bromide (EtBr) showing the results of (A) ApoI digest of pFastBac1 wtIGFBP-5 and G203K cDNAs (B) SphI digest of pFastBac1 wtIGFBP-5 and Q209A cDNAs. SM represents DNA size markers (λ-HindIII and $\phi\chi174$ in A; λ-HindIII alone in B), and fragment size is indicated. Arrows indicate newly created restriction fragments in the mutants.

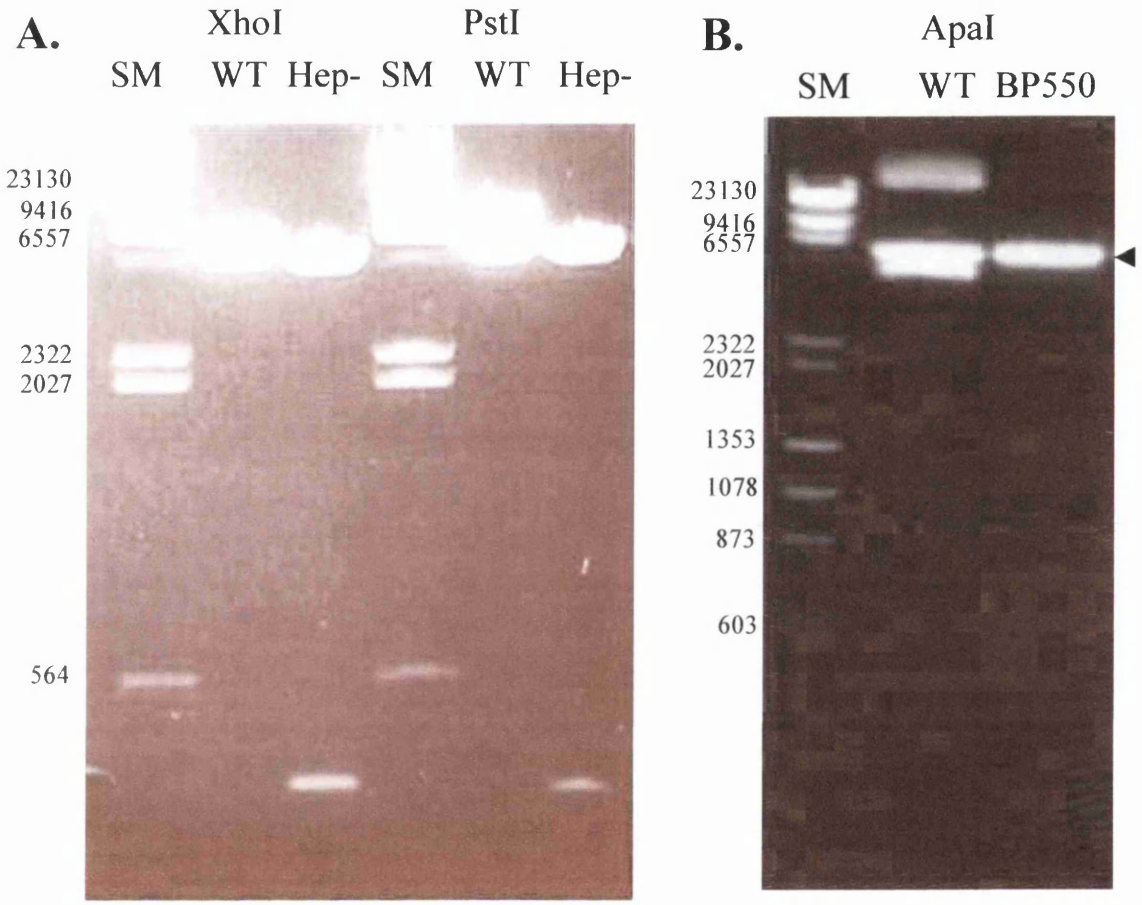


Figure III.5 Restriction enzyme analyses of wild type (WT) IGFBP-5, Hep-, and BP550 inserted in pFastBac1 plasmid (A) XhoI digests of wtIGFBP-5 and Hep- and PstI digests of wtIGFBP-5 and Hep-. (B) ApaI digest of the pFastbac1 wtIGFBP-5 and BP550. SM represents DNA size markers (λ-HindIII alone in A and; λ-HindIII and $\phi\chi174$ in B), and fragment size is indicated. Arrows indicate newly created restriction fragments in the mutants.

from the IGFBP-5 sequence and 58bp from pFastBac1) (Figure III.5A). The BP550 mutation introduces a unique ApaI restriction site, as there are no ApaI restriction sites in either the IGFBP-5 or pFastBac1 plasmid sequence (note that this restriction site is *dcm* methylation sensetive, so that the mutant DNA had to be amplified in the JM110 bacterial cell line, which is *dcm* free). As expected, ApaI does not cut pFastbac1WTIGFBP-5, whereas pFastBac1BP550 is linearised (Figure III.5B).

III.2.2 Expression of mutant proteins

The baculovirus expression system was chosen to express wt IGFBP-5, G203K, Q209A, Hep-, Double and BP550 in insect cells. The baculovirus expression system offers the advantage of being able to directly harvest the recombinant protein in a soluble form from the insect cell conditioned medium, as these cells are able to utilize mammalian signal peptide sequences and secrete the protein.

Following the construction of recombinant baculoviruses carrying either wt IGFBP-5 or other mutant DNAs, the expression of the encoded proteins was obtained by infecting Sf9 insect cells, as described in chapter II. To detect the presence of WTIGFBP-5 or other mutant proteins in the insect cell supernatants, equal volumes of the concentrated conditioned media were collected following a time-course and electrophoresed on a 12% (or 16% for BP550) gel under non-reducing conditions. Figure III.6 shows the results of IGFBP-5 western blots with these samples. From this work, it became apparent that the virus infection time required for achieving optimal yield of recombinant protein varied between the different clones. WTIGFBP-5 and the other mutant proteins appeared as a major band of approximately 34kDa, with the exception of BP550 at 24kDa (Figure III.6). Others have reported an IGFBP species expressed from sf9 insect cells, which had IGF binding affinities similar to mammalian IGFBP-1 (Doverskog et al, 1999). However, we have tested our sf9 cells, which were obtained directly form Gibco-Life technology, and two independent researchers in our group were un able to detect any IGF binding activity in culture medium from uninfected cells (Figure III.7; pers. Comm. S. Bramani). We consider it unlikely that different strains of sf9 insect cells would differentially express an IGFBP species, and believe that our results question the validity of the data of Doverskog et al, 1999.

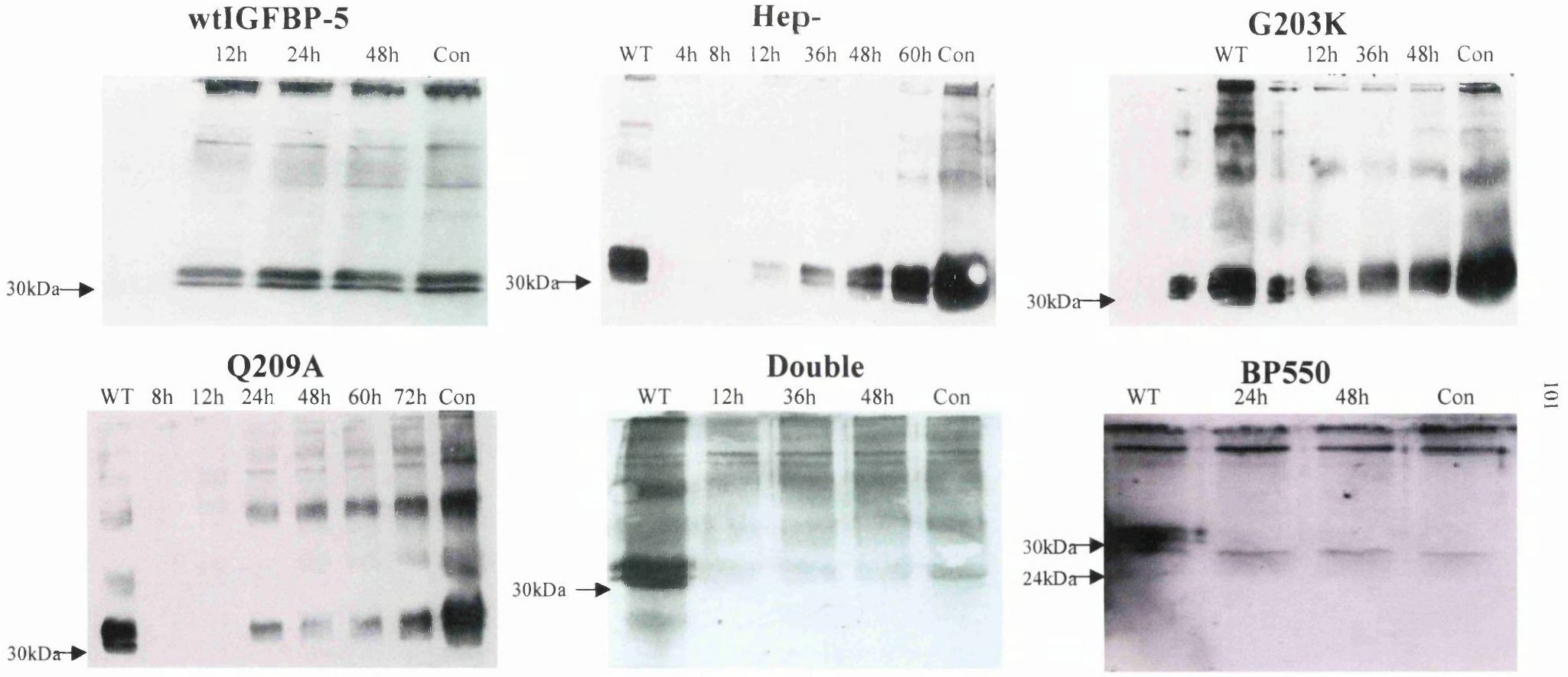


Fig III.6 Time course of protein expression using the baculovirus expression system. Detection of protein expression in transfected insect cell culture medium. Proteins were collected following a time-course (h indicates hours post-infection), and concentrated (Con) ten-fold using 'Ultrafree-20' centricon columns (Millipore, Boston, USA). 20µl of each sample were analysed by western immuno blotting, using the anti-rat IGFBP-5 antisera as the primary and anti-sheep HRP as the secondary.

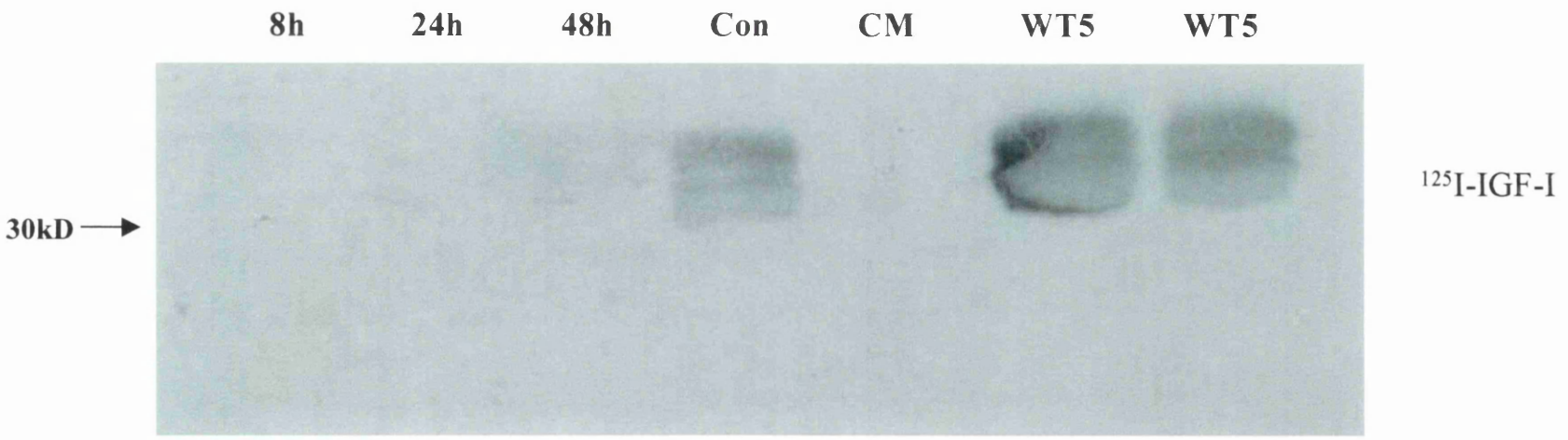


Fig III.7 ^{125}I-IGF-I ligand blot analysis of Hep- time course expression including CM (conditioned medium - uninfected sf9 insect cell culture medium). 15µl of each sample were analysed by ^{125}I-IGF-I ligand blotting. Two separate preparations of WTIGFBP-5 were run in the above gel.

Following concentration, the harvested proteins were purified on an IGF-II affinity column (described in chapter II). The purity of the proteins were assessed on IGFBP-5 westerns, and the results for WTIGFBP-5 are shown in Figure III.8. All the recombinant proteins described in this thesis were subject to the same purification procedure and showed immunogenic reactivity with either anti-rat IGFBP-5, or anti-rat IGFBP-2 (as described in chapter IV). Although there was some non-specific loss of protein during the washing stages, we were able to obtain protein concentrations of 40, 46, 45, 25, 22 and 24ug/ml for WTIGFBP-5, G203K, Q209A, Hep-, Double, and BP550 respectively, as measured by Bradford assay. The *in vitro* analyses of these purified proteins forms the remainder of the results in this chapter.

III.2.3 Analysis of G203K and Q209A

Western ligand and immunobloting. Approximately 200ng of WTIGFBP-5, Hep- and the two single mutants, G203K and Q209A, were loaded on a 12% polyacrylamide gel under non-reducing conditions to examine the binding to both ^{125}I-IGF-I and –II by ligand blot analysis. Following ligand blot analysis, the membrane was re-probed with anti-rat IGFBP-5 antisera to monitor protein loading. Figure III.9A shows that, although both G203K and Q209A are slightly overloaded with respect to WTIGFBP-5 and Hep-, the binding of both G203K and Q209A to IGFs is clearly compromised compared with WTIGFBP-5. Scanning densitometry of these blots indicates that binding of G203K and Q209A was reduced to approximately 15-25% of that seen for the WTIGFBP-5 for both IGF-I and IGF-II binding (Figure III.9B). Conversely, the binding of the Hep- mutant to either ^{125}I-IGF-I or –II was not affected, giving a signal on ligand blots equivalent to that seen for WTIGFBP-5. This experiment was repeated six times with similar results in each instance.

Solution phase analysis was performed to examine the affinity of these four proteins for IGFs (FigureIII.10). Data were analysed by Scatchard transformation (Scatchard, 1949) which generated a linear regression binding curve. Scatchard analysis of the binding curves for WTIGFBP-5, Hep-, G203K and Q209A proteins gave Kd values of 0.061, 0.058, 0.283 and 0.173nM respectively for binding to IGF-I (Figure III.10A) and 0.024, 0.41, 0.077 and 0.12nM

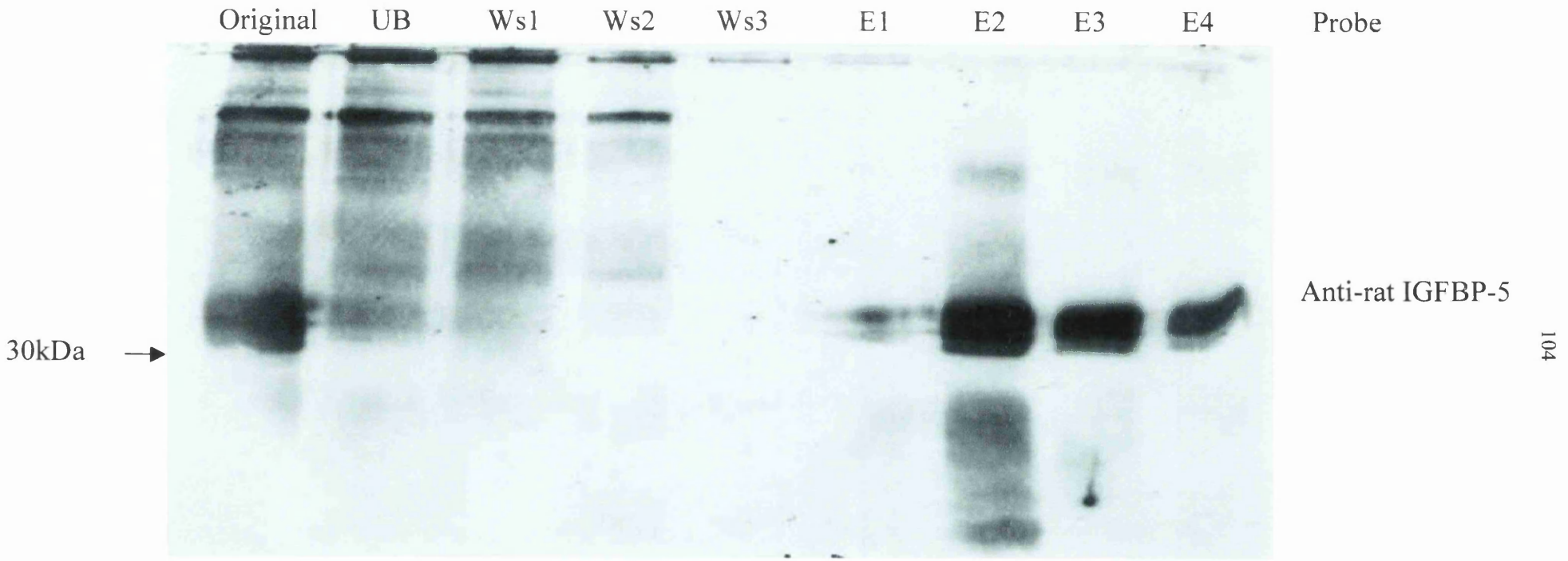


Fig III.8 IGF-II affinity chromatography purification of wtIGFBP-5.Concentrated insect cell culture supernatant (Original) was loaded onto the IGF-II affinity column, the unbound material (UB) was collected and the column was washed (Ws) three times with the equilibration buffer. Elution (E) was performed with 0.5M acetic acid and the first 4 fractions were analysed for the presence of IGFBP-5. Samples were taken from each purification step and electrophoresed on a 12% gel under non denaturing conditions and proteins were detected by western immuno blotting. In house anti-rat IGFBP-5 was used for primary probe and anti-sheep HRP was used as secondary probe.

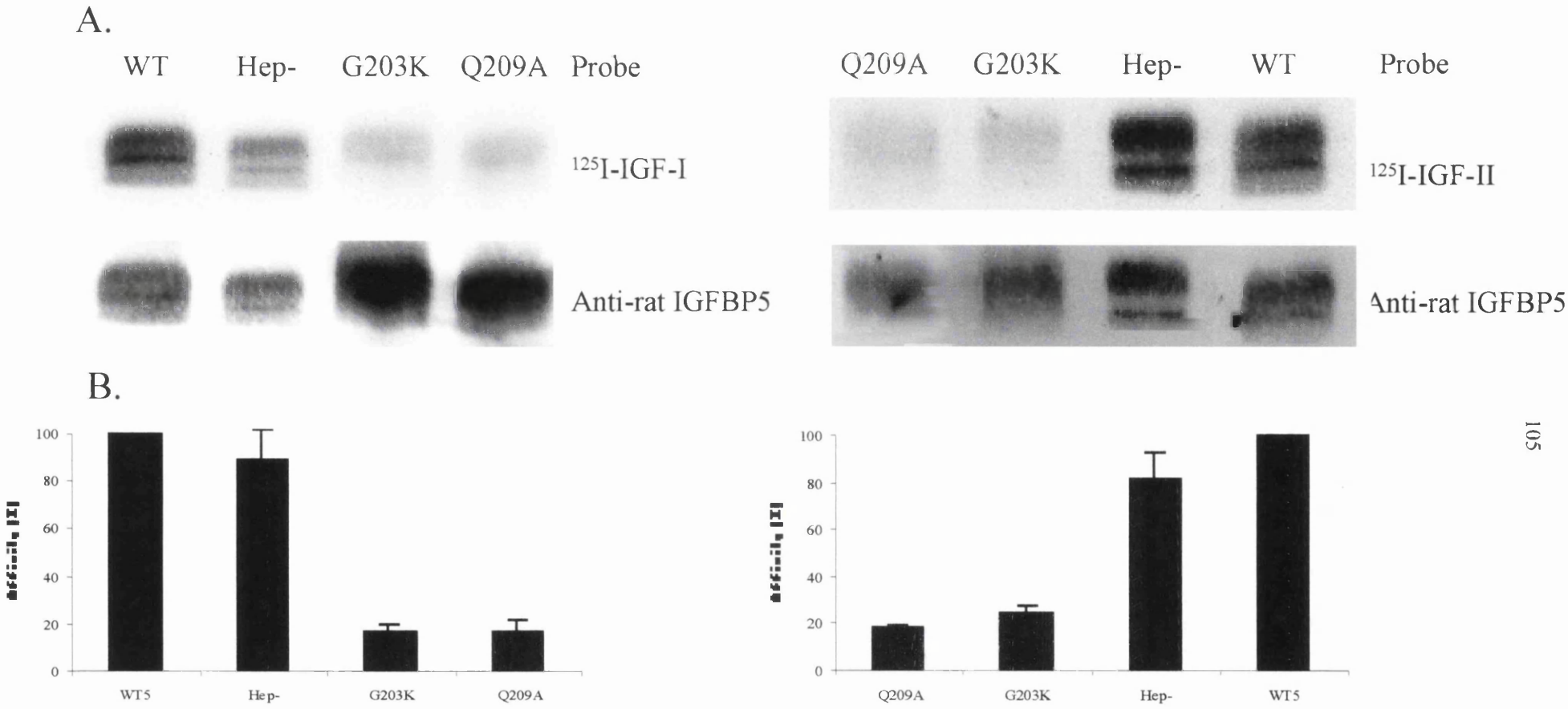


Fig III.9 (A) ^{125}I-IGF-I and -II ligand blots of wild-type (WT), G203K, Q209A and Hep- proteins. Ligand blotting was performed as described in chapter II. Protein loading levels were monitored by probing the same blots with polyclonal anti-rat IGFBP-5 antibodies (lower panel) after development of ligand lots. (B) Densitometric analysis of ligand-binding data. Levels of binding are expressed as mean (4 or 5 times of experimental value) of band intensity ± Standard deviation; (ligand blot/western blot) X 100%. For binding IGF-I, Hep-:89.2±12.9, G203K: 16.7±2.8, Q209A: 16.8±4.7, for binding IGF-II, Hep-81.3±11.7, G203K: 24.0±3.3, Q209A: 18.5±1.3.

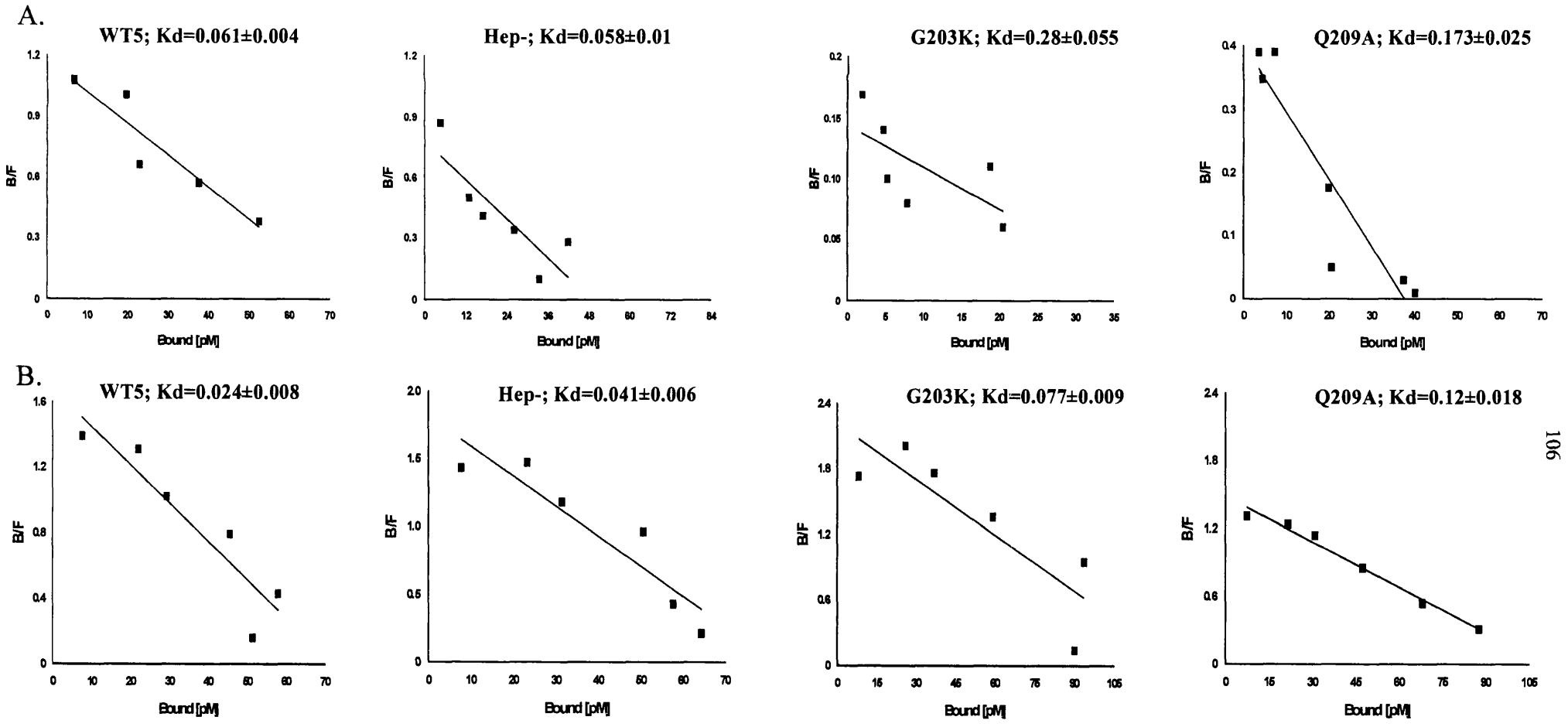


Fig III.10 Solution-phase assay of IGFBP activity. Wild type (WT), G203K, Q209A and Hep- were analysed by solution-phase assay as described in the chapter II. Scatchard analysis was derived after the determination of IGFBP binding to (A) ^{125}I-IGF-I or (B) IGF-II in the presence of increasing concentrations of unlabelled IGF-I or -II. In both instances, regression lines are drawn through the data obtained by Scatchard analysis. B/F, bound/free. Kd=Mean (Kd) ± Standard Error. The mean Kd values in this figure were derived from 3 or 4 separate experiments with the individual clones.

respectively for binding to IGF-II (Figure III.10B). These data for WTIGFBP-5 indicate a 2.5-fold greater affinity for IGF-II than for IGF-I, which agrees with the ligand preference reported for human IGFBP-5 (Kiefer *et al*, 1992). These data also indicate that the compromised binding of IGF-I and -II to G203K and Q209A detected by ligand blot was also apparent in solution phase, with a 4.6- and 3.2-fold reduction in binding of G203K to IGF-I and –II, respectively and a 2.8- and 5-fold reduction in Q209A binding to IGF-I and –II, respectively. We did not detect any significant differences in binding IGFs between WTIGFBP-5 and the Hep- mutant by either ligand blotting or solution phase assay. These experiments were repeated four times with similar results obtained in each instance.

III.2.4 Analysis of Double mutant and BP550

Although the G203K and Q209A mutantions caused a significant reduction in binding to both IGF-I and –II, this was not comparable with the previous C-terminally truncated IGFBP-2 mutant of Forbes *et al.* (1998) where they had observed an 80-fold reduction in binding IGF-II following the deletion of both of these amino acids. Consequently, we decided to mutate both amino acids to establish whether there might be a cumulative effect of G203 and Q209 on IGF binding. Figure III.11 shows the results of an ^{125}I-IGF-I and –II ligand blot and an anti-IGFBP-5 immuno blot for WTIGFBP-5, Hep- and Double mutant. Our ligand blots demonstrate that binding of Double mutant to ^{125}I-IGF-I is completely ablated , where ^{125}I-IGF-II binding is minimal (Figure III.11). This experiment was repeated five times with similar results in each instance. For further confirmation of the IGF-binding properties of the Double mutant, solution phase analysis was performed with both Double mutant and BP550. Data were again analysed by Scatchard. Kd values of Double and BP550 were 5.01 and 3.86nM for binding to IGF-I (Figure III.12A), and 4.8 and 4.1nM for binding to IGF-II respectively (Figure III.12B). Comparing this to the affinity of WTIGFBP-5 (Figure III.10) shows that the Double mutant has a 82- and 200-fold lower affinity for IGF-I and –II, respectively, whereas BP550 has a 63- and 170-fold lower affinity for IGF-I and –II respectively. This experiment was repeated four times, with similar results obtained each time.

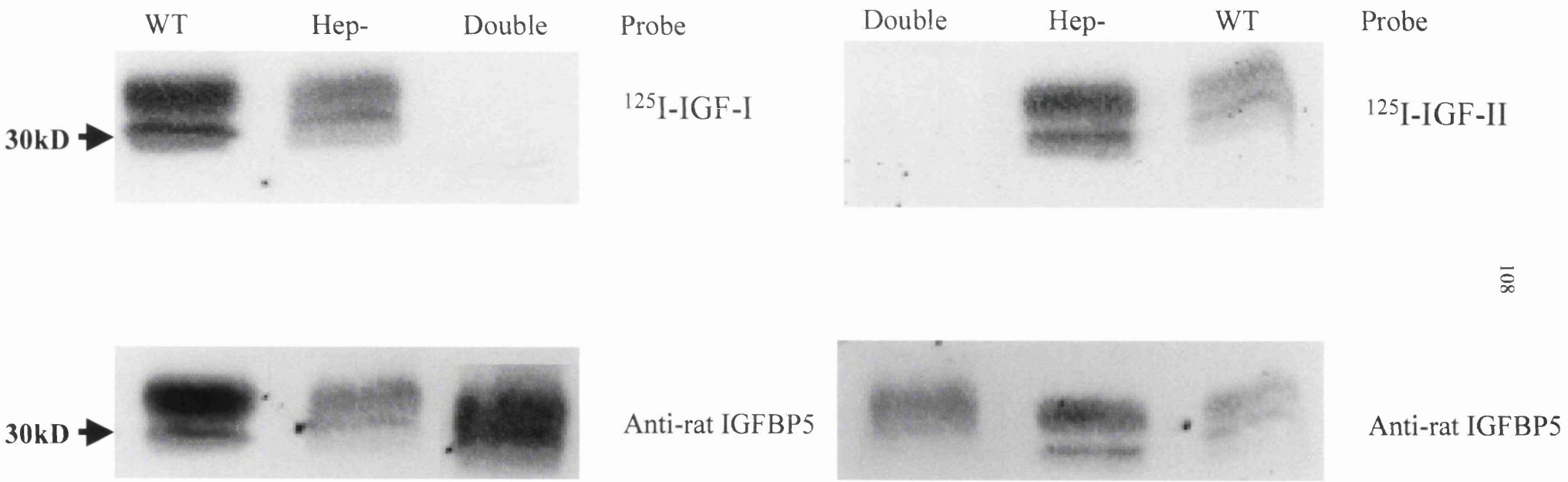
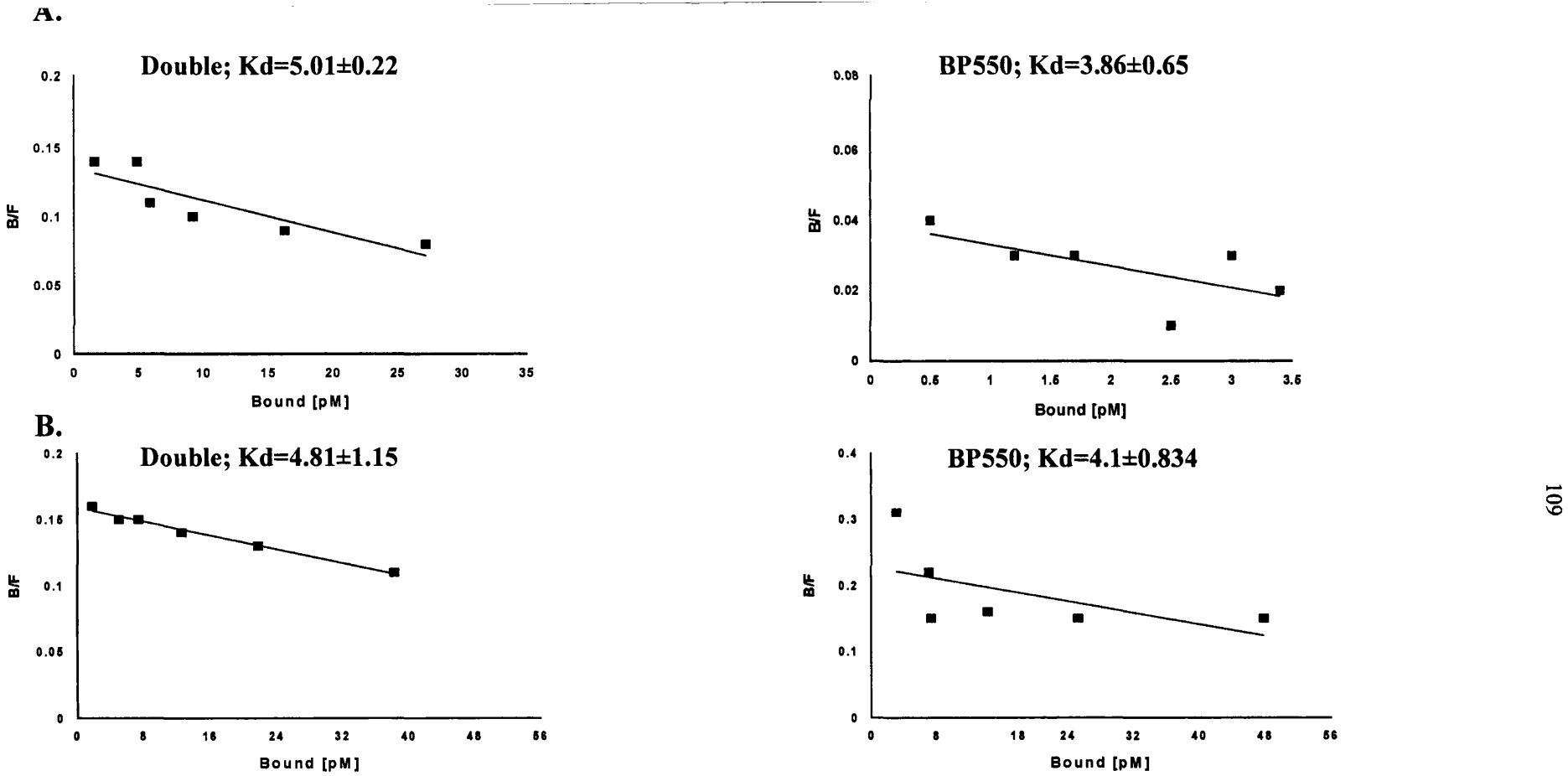


Fig III.11 ^{125}I-IGF-I and -II ligand blots of wild-type (WT), Hep-, and Double proteins. Ligand blotting was performed as described in chapter II. Protein loading levels were monitored by probing the same blots with polyclonal anti-rat IGFBP-5 antibodies (lower pannel) after development of ligand lots.



III.12 Solution-phase assay of IGFBP activity. Double and BP550 were analysed by solution-phase assay as described in chapter II. Scatchard analysis was derived after the determination of IGFBP binding to (A) ^{125}I-IGF-I or (B) IGF-II in the presence of increasing concentrations of unlabelled IGF-I or -II. In both instances, regression lines are drawn throuhg the data obtained by Scatchard analysis. B/F, bound/free. Kd=Mean (Kd) ± Standard Error. The mean Kd values in this figure were derived from 4 separate experiments.

III.2.5 BIA-core analysis

As a further confirmation of the IGF-binding properties of these IGFBP-5 mutant proteins, biosensor analysis of binding kinetics of the proteins were undertaken to both immobilised IGF-I and –II biosensor surface (Table III.1). This type of analysis gives details of on- and off-rate kinetic constants from which association and dissociation constants can be derived, whereas solution phase assay only represents equilibrium binding events. The qualitative differences in the affinity of the four IGFBP-5 proteins for both IGF-I and –II were maintained (Table III.1 and Figure III.13). Closer inspection of the Biacore data revealed that the reduction in the affinity of the mutants for IGF-I and IGF-II was due largely to a reduction in association rates, with much smaller effects on the dissociation rates for these complexes. Therefore the overall equillibrium constant (K_D) of G203K showed a 6.5-and 15-fold lower affinity than that of WTIGFBP-5 in binding IGF-I and –II respectively. For the Q209A mutant, there was a 12- and 51-fold reduction compared to WTIGFBP-5 in binding IGF-I and IGF-II respectively. For the Double mutant, no real association could be measured in this system. However note that both ligand and western immuno blot showed a doublet band of the same size at WTIGFBP-5, which bound ^{125}I-IGF-II weakly. The C-terminally truncated BP550 also showed large reductions in binding to IGF-I and IGF-II, although the effect for IGF-I was less than that for IGF-II.

Also of interest was the observation that, although the overall affinity (K_D) of the Hep- mutant for IGF-I and –II was similar to that of wild-type IGFBP-5 at the level of rate constants there were apparent differences for the interaction of Hep- with either IGF-I or IGF-II. However, the 8- and 26-fold decreases in K_{ON} values for Hep- binding to IGF-I and –II, respectively, were partly compensated by the 15- and 67-fold decreases in K_{OFF} values obtained for the same binding events. This results in a similar affinity for WTIGFBP-5 and Hep- binding to IGF-I and –II. In addition, the absolute values for equilibrium constants (K_D) derived by biosensor were two to three orders of magnitude lower than that obtained with the charcoal-based solution phase assays (see Discussion). The BiaCore experiments were carried out on two separate occasions, with extremely similar data produced on both occasions.

Table III.1 Bia-core analysis of kinetic constants for WTIGFBP-5, hep-, G203K, Q209A, Double and BP550 binding to immobilised IGF-I and –II. For further details, see chapter II.

Protein	IGF-I			IGF-II		
	K_{ON} (Ms^{-1})	K_{OFF} (s^{-1})	K_D (M^{-1})	K_{ON} (Ms^{-1})	K_{OFF} (s^{-1})	K_D (M^{-1})
WTIGFBP-5	4.9×10^{4}	1.6×10^{-3}	3.2×10^{-8}	2.5×10^{5}	3.3×10^{-4}	1.3×10^{-9}
Hep-	6.3×10^{3}	1.1×10^{-4}	1.8×10^{-8}	9.7×10^{3}	4.9×10^{-5}	5.0×10^{-9}
G203K	9.4×10^{3}	2.0×10^{-3}	2.1×10^{-7}	2.8×10^{4}	5.7×10^{-4}	2.0×10^{-8}
Q209A	1.7×10^{4}	6.8×10^{-3}	3.9×10^{-7}	6.3×10^{3}	5.2×10^{-4}	6.5×10^{-7}
Double	0.0735	3.7×10^{-3}	0.0504	759	5.7×10^{-3}	7.5×10^{-6}
BP550	1.6×10^{3}	0.0173	1.1×10^{-5}	2.1×10^{3}	3.6×10^{-3}	1.7×10^{-6}

$K_D = K_{OFF}/K_{ON}$

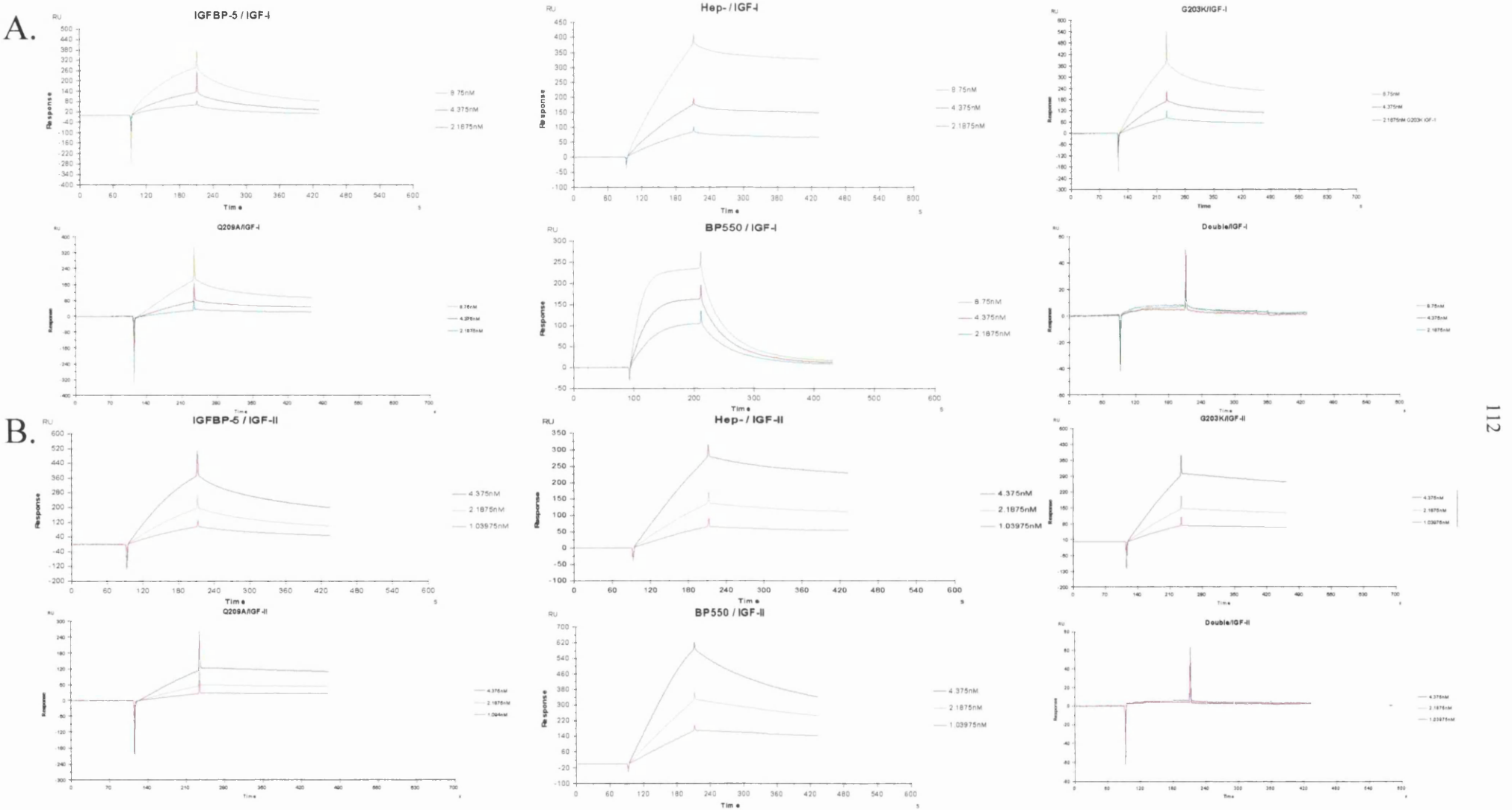


Fig III.13 Individual sensograms for wtIGFBP-5, Hep-, G203K, Q209A, Double, and BP550 binding to both human IGF-I (A) and IGF-II (B) ligands immobilized on the surface of biosensor chips. The sensograms show real time K_{ON} and K_{OFF} values with three different concentrations of IGFBPs (top, middle and bottom line corresponding to 8.75nM, 4.375nM and 2.1875nM respectively with IGF-I; top, middle and bottom line corresponding to 4.375nM, 2.1875nM and 1.03975nM respectively with IGF-II).

III.2.6 Heparin binding assay

Experiments were also carried out to investigate the heparin-binding properties of WTIGFBP-5 and other mutant proteins. Using a blotting methodology with a heparin-biotin conjugate and streptavidin-HRP/ECL detection system, we were able to confirm that the Hep- mutant containing mutations at four basic residues important for heparin binding (R201L, K202E, K206Q and R214A) showed greatly reduced heparin binding (Figure III.14). However, the G203K, Q209A and Double mutant displayed a binding activity for the heparin-biotin conjugate similar to that displayed by the WTIGFBP-5. This experiment was repeated seven times, with similar results in each instance.

III.3 Discussion

The IGFBP-5 doublet produced by expression in the baculovirus system is a common feature from several laboratories (personal communications Dr. D. Andress, Dr. S. Guenette), including commercially available hIGFBP-5 (Austral biologicals), and this may be the result of inefficient cleavage of the mammalian signal peptide during secretion. In addition, others have observed an IGFBP-5 doublet expressed from human fibroblast cells (Arai *et al.* 1994b), demonstrating that this is not specifically a result of expression of IGFBP-5 in insect cells. Nevertheless, Biacore analysis of the wild type rIGFBP-5/hIGFs interaction recorded a single association and dissociation event suggesting that the IGFBP-5 doublet represents a single species with respect to IGF binding. Also, the strong recognition of the mutant proteins with our in house IGFBP-5 antibody demonstrates their immunological integrity.

Ligand blot analysis with ^{125}I-IGF-II followed by densitometry indicated binding of G203K and Q209A at 15-25% of that seen for wt IGFBP-5, which agreed with the data obtained following ligand blot analysis with ^{125}I-IGF-I. However, solution phase assay with IGF-II indicated a somewhat smaller decrease in affinity - reduced 3-fold and 5-fold for G203K and Q209A respectively. Although we did not conduct a systematic analysis of the differences in ligand binding between these two methodologies such differences have been reported previously (Hobba *et al*, 1998). In a similar vein, ligand blot analysis suggested equivalent binding of labeled IGF-I and -II

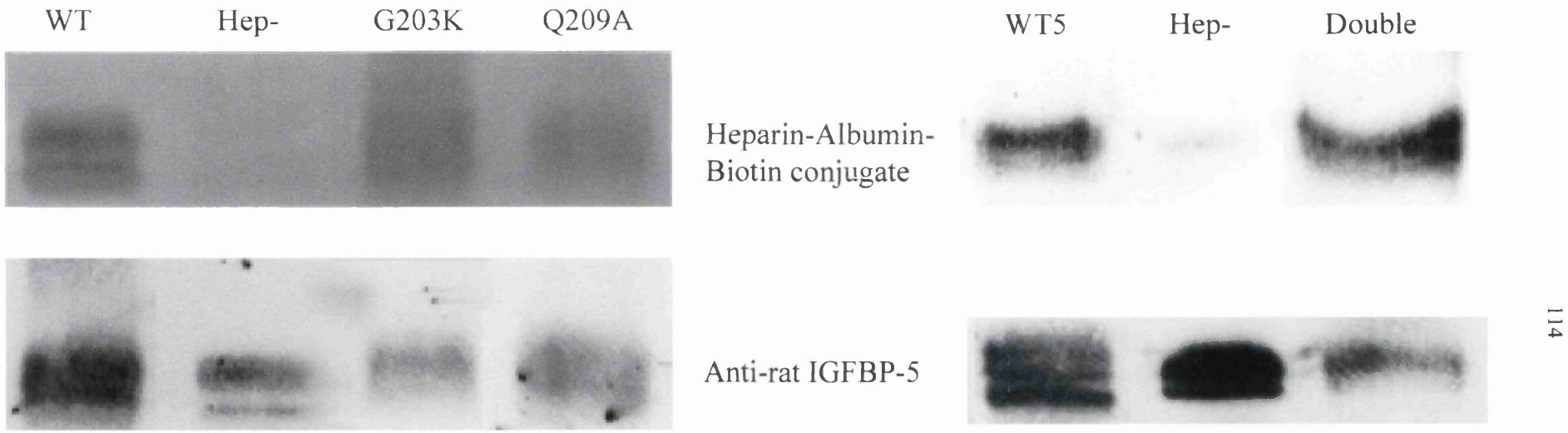


Fig III.14 Heparin ligand blot of wild type (WT), G203K, Q209A, Hep-, or Double proteins (upper pannel) was performed as described in chapter II. Heparin-Albumin-Biotin conjugates were used for primary probe, and streptavidin-HRP was used for secondary probe. As for ligand blots described in Fig III.6, protein loading was monitored by western blotting of duplicate filters with anti-rat IGFBP-5 antisera. A representative autoradiograph shows the clearly diminished heprin binding of the Hep- protein.

by WTIGFBP-5 and Hep- proteins. This was paralleled by only a small difference in affinity between the two proteins on solution phase analysis of binding (Hep- has same and 1.7-fold lower affinity for both IGF-I and -II). Interestingly, BiaCore analysis of Hep- indicated a much reduced value for Kon compared to WTIGFBP-5. However this was balanced by the fact that Hep- also displayed a lower Koff when compared to WTIGFBP-5. It has been suggested by Hobba *et al* (1998) that ligand blot analysis may rank IGF binding to IGFBPs in order of the K_{OFF} values for complex formation. Although this is still open to some debate our ligand blot data show equivalent signals for WTIGFBP-5 and Hep- proteins. It is important to note that the Kd of our WTIGFBP-5, as determined by solution phase assay is in agreement with the work of others using mammalian IGFBP-5 and the yeast expressed protein (Kiefer *et al.* 1992).

In a qualitative sense our biosensor data support the findings of both the ligand and solution phase assays of IGF affinity with major effects of G203K and Q209A binding to IGF-I and -II and a much smaller effect of Hep- interaction with either of the IGFs, although there are specific effects on K_{ON} and K_{OFF} for this latter protein (see Results). The absolute Kd values obtained by Biosensor are 10-100 fold lower than those seen in solution phase and are also lower by the same order of magnitude than the affinity constants reported for the human IGFBP-5 interaction with hIGF-I by BiaCore (Kalus *et al*, 1998). However, these researchers used biotinylated IGF-I or -II immobilised to streptavidin coated biosensor chips together with different conditions for monitoring on and off rates. In addition the homologous interaction system reported by these authors (human IGFs and human IGFBP-5) is different from the current study where rat IGFBP-5 proteins were exposed to sensor chips containing directly immobilised human IGF-I or -II. Together, these experimental differences may account for the different results obtained. Interestingly, and supportive of this argument, are the findings of Hobba *et al*, 1998, who examined the heterologous interaction of bovine IGFBP-2 with immobilised human IGF-I and -II. In this study conditions of ligand immobilization and binding analysis were similar to those described in the current work and accordingly these authors reported kinetic constants of the same order as reported here. Notwithstanding these arguments, our Biosensor data fully supports our ligand blotting and solution phase data indicating compromised binding of G203K and Q209A to both IGF-I and IGF-II with only a relatively minor affect on IGF binding of Hep-. In conclusion, we have demonstrated that

Gly203 and Gln209 in rIGFBP-5 are important determinants in binding IGFs as measured by three independent techniques, ligand blotting, solution phase assay and Biacore analysis.

Others have found a far greater reduction (upto 80-fold) in IGF-II affinity when the equivalent amino acids (222-236) were deleted from bovine IGFBP-2 (Forbes *et al.* 1998). This may reflect differences in the C-terminus between IGFBP-5 and –2 with respect to binding IGF-II, or alternatively, the loss of both of these residues, as is the case with the IGFBP-2 truncation, may have a cumulative effect which results in a greater reduction in binding. In order to test this hypothesis, we subsequently made both the Double (G203K/Q209A) and C-terminally truncated BP550 mutants. As the C-terminal domain of IGFBP-5 has been previously shown to be required for IGF binding, (Andress *et al*, 1993), BP550 serves as a negative control which cannot bind ligand effectively. As expected, ligand blotting could not detect any binding of BP550 to IGF-I and only very weak binding activity to IGF-II. Interestingly, this was also the case for the Double mutant. In support of this, our solution phase experiments demonstrated that BP550 had a 63- and 170-fold reduced Kd for IGF-I and –II respectively, compared to WTIGFBP-5 (Figure III.12), whereas the Double mutant had a 82- and 200-fold reduction in binding affinity for IGF-I and –II respectively. Furthermore, in the Biacore assay, the Double mutant showed a large reduction in binding both IGF-I and IGF-II and again this was manifested as a major decrease in the association rate for the proteins, whereas the dissociation rate was relatively less affected. Therfore, we have clearly shown that there is a cumulative effect of Gly203 and Gln209 on IGF binding, where loss of both leads to a far greater reduction in IGF binding affinity, and this may explain the previous discrepancy with the data of Forbes *et al* (1998) for IGFBP-2. In addition, as the Double mutant has a reduction in IGF binding that is comparable to that for BP550, which lacks the entire C-terminal domain, this may indicate that Gly203 and Gln209 are the major determinants in this domain with respect to IGF binding.

The fact that Gly203 and Gln209 in the C-terminus of IGFBP-5 are completely conserved between all six binding proteins from all species sequenced to date suggests that they may have a conserved function in IGFBP biology. From this work it would appear that for IGFBP-5 at least, one of the functions of these residues involves IGF binding. This is of major significance when one considers that in IGFBP-5 and –3 these two residues lie in the middle of a stretch of basic amino

acids that have previously been shown to be involved in binding to heparin and to components of the ECM (Arai *et al.* 1996a; Parker *et al.* 1996). Other functions ascribed to this region in IGFBP-5 include binding to the acid labile subunit (Twigg and Baxter, 1998), the putative IGFBP-5 receptor (Andress 1998), and plasminogen activator inhibitor-I (Nam *et al.* 1997). It has even been postulated that this sequence has strong homology to nuclear localization signals (Schedlich *et al.* 1998) and the DNA-binding domains of several transcription factors (Schedlich *et al.* 1998; Mitchell & Tjian 1989).

Heparin ligand analysis clearly demonstrates that the binding of mutants G203K, Q209A and Double for heparin is not compromised (Figure III.14). However, as a control we also decided to study some of the flanking basic amino acids, mutation of which had been shown by others to affect heparin binding (Arai *et al.* 1996a), while not affecting IGF binding (Parker *et al.* 1998). This is confirmed with the results for our mutant Hep- (R201L, K202E, K206Q, R214A), which had a major reduction in binding heparin, but unimpaired binding to IGFs (Figure III.9, 10, 13, & 14). Therefore, it would appear that in the linear sequence, basic amino acids involved in heparin binding are interspersed with non-basic amino acids involved in IGF binding. A helical wheel prediction of the 201-218 region of IGFBP-5, places Gly203 adjacent to important ECM binding residues, while Gln209 is flanked by two further basic residues elsewhere in the wheel (Figure III.15). This demonstrates that there may be overlap between IGF and ECM binding sites in the C-terminus of IGFBP-5.

As Gly203 and Gln209 are conserved in all six binding proteins, whereas the C-terminal heparin binding sequence is only common to IGFBP-5 and –3, this may mean that the IGF binding function of this region predates heparin binding. Therefore, the heparin/ECM binding sequences in IGFBP-5 and –3 may have evolved later to provide greater regulation of an increasingly complex IGF system. Whether this regulation serves to potentiate or inhibit the effects of IGFs is unresolved, although experiments from other laboratories clearly indicate that heparin/ECM bound IGFBP-5 has between an 8- and 17-fold reduced affinity for binding IGF-I (Andress & Birnbaum 1992; Jones *et al.* 1993; Arai *et al.* 1994a). These reduced affinities may be crucial in obtaining the correct balance between soluble IGFs available for binding IGF receptors, and ECM bound IGFBP-IGF complexes.

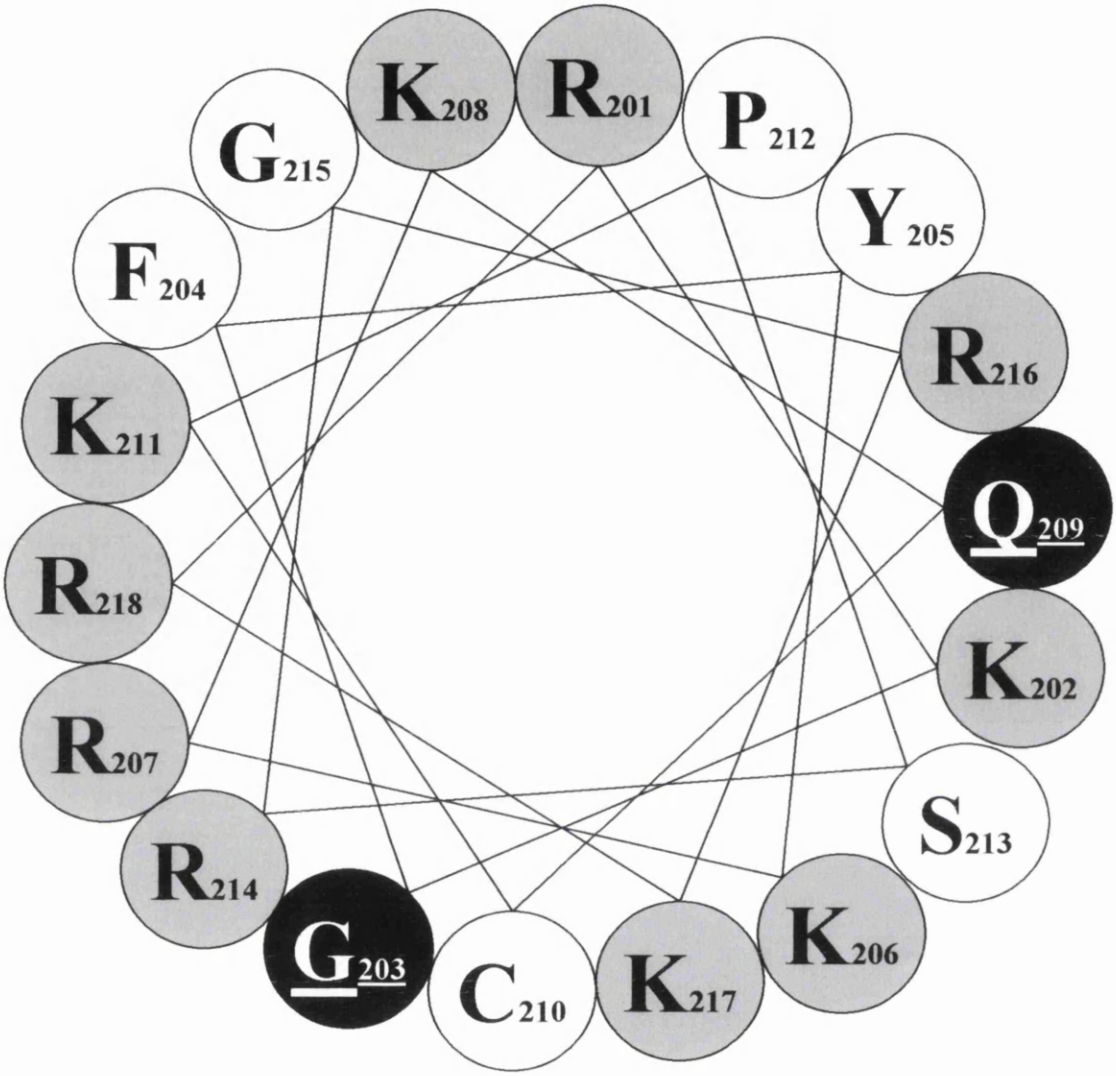


Fig III.15 Helical-wheel projection of the 201-218 region of rat IGFBP-5. The location of basic R and K residues believed to participate in heparin binding are indicated by grey shading, and the residues Gly203 and Gln209 (mutated in this study) are shown in black. (adopted from Parker *et al*, 1998)

One explanation offered for the reduced affinity of ECM bound IGFBP-5 for IGF-I is that heparin binding might induce a conformational change in IGFBP-5, which alters an IGF binding domain elsewhere in the protein (Arai *et al.* 1996a) (Figure III.16A). Based on the work reported here, where we indicate a potential overlap between heparin and IGF binding sites in the C-terminal domain of rat IGFBP-5, we propose an alternative more direct model whereby heparin or ECM binding to the basic amino acids in the 201-218 region of IGFBP-5 may physically interfere with the subsequent interaction with IGFs (Figure III.16B). It is possible however that this region of the IGFBP-5 protein does not exist as a helical structure and that there is a spatial separation of G203, Q209 and the important basic residues. Further advances in this area will be achieved by the careful structural and functional analysis of heparin-IGFBP5-IGF complexes.

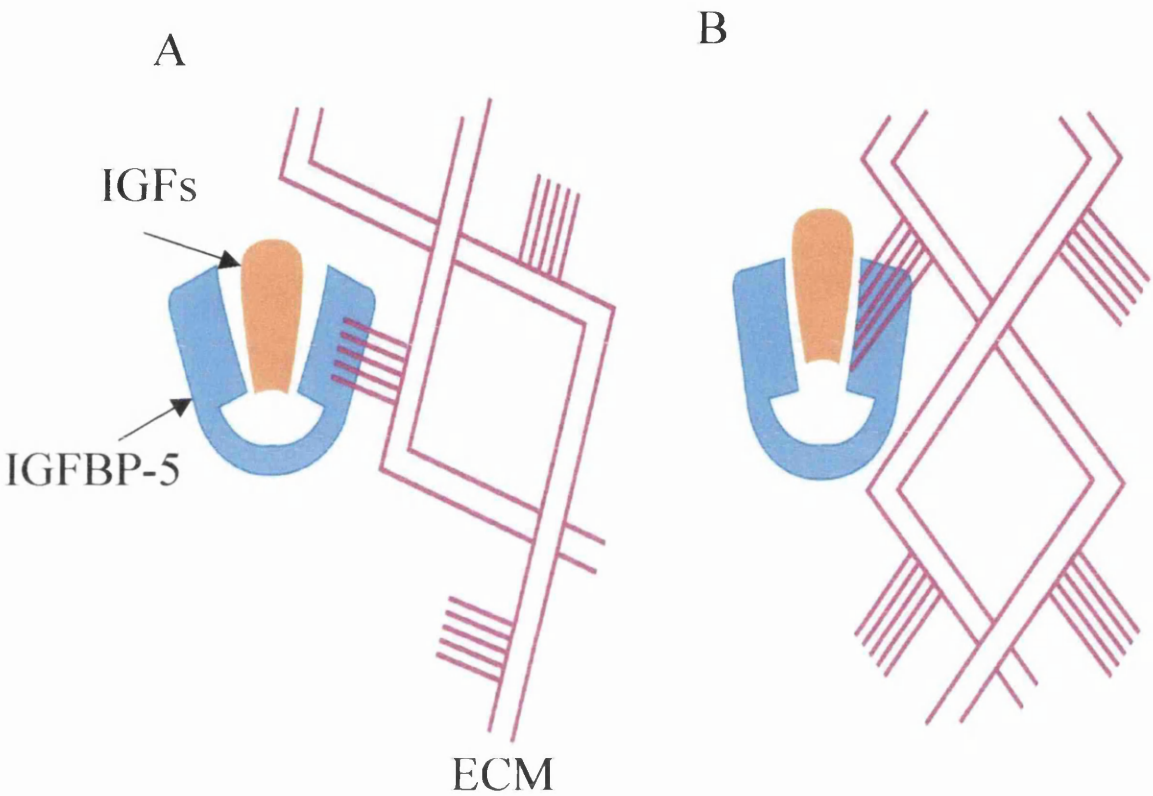


Fig III.16 Schematic diagram outlining two models for the interaction of IGFBP-5 with components of the extracellular matrix and the subsequent lowering of affinity for IGF-IGFBP-5 interaction. In (A) binding of IGFBP to components of the ECM results in a conformational change in IGFBP structure such that the affinity of the binding protein for ligand is decreased. In (B) , as supported by the data from the current study, there is overlap between the IGF and ECM binding domains in IGFBP-5, offering a more direct route by which interaction of IGFBP-5 with ECM may affect subsequent IGF binding.

Chapter IV. Binding of chimeric IGFBP-2 and –5 proteins to IGFs

IV.1 Introduction

As discussed in chapter I.3.3, many researchers have performed experiments aimed at identifying the IGF binding motifs in the N- and C-terminal domains of IGFBPs (Hobba et al, 1996; Kalus et al, 1998; Imai et al, 2000; Song et al, 2000). Although, progress has been made in identifying the specific amino acids, that are involved in IGF binding, the exact contribution of the entire N- and C-terminal domains of IGFBPs in IGF binding is still controversial. For example, Kalus et al. (1998) have proposed that the primary IGF binding site is in the N-terminal domain and that the C-terminal domain merely serves to stabilise this primary binding event. It has been generally accepted that the two highly structured terminal domains are likely to be in simultaneous contact with the smaller IGF molecule, with the more flexible central domain connecting them, although this model will only be confirmed by the eventual determination of IGFBP-IGF structure through techniques such as X-ray crystallography.

In addition, ligand preferences of IGFBPs have been reported (reviewed by Jones and Clemmons, 1995; Clemmons, 1997). How this ligand preference operates at the level of the terminal domains is not known. One suggestion that this preference may operate in the C-terminal domain of IGFBP-2 came from the work of Forbes et al. (1998), where they found that the affinity for IGF-II was affected far more than that for IGF-I binding in C-terminally truncated mutants of this binding protein. Contrary to this, there are several reports from other laboratories that a proteolytic N-terminal IGFBP-5 fragment binds to IGF-II (Imai et al, 1997; Hakeda *et al*, 1996), whereas 13.3 and 13.5Kd C-terminal fragments retain significant IGF-I binding (Standker *et al*, 1998).

In order to assess the contribution of the N- and C-terminal domains to IGF binding, we decided to make chimeric IGFBP proteins by switching domains between rat IGFBP-5 and rat IGFBP-2. In the context of IGF binding, these two binding proteins seemed the best choice from which to make chimeras, due to their very marked differences in IGF binding. IGFBP-2 and IGFBP-5 have been reported to have the lowest and highest affinities for binding IGFs respectively

among all six IGFBPs, although both have a preference for binding IGF-II over IGF-I (Kiefer *et al.*, 1992). In addition, IGFBP-5 and –2 differ greatly with respect to binding heparin/ECM and the cell surface and this will be dealt with in more detail in chapter V.

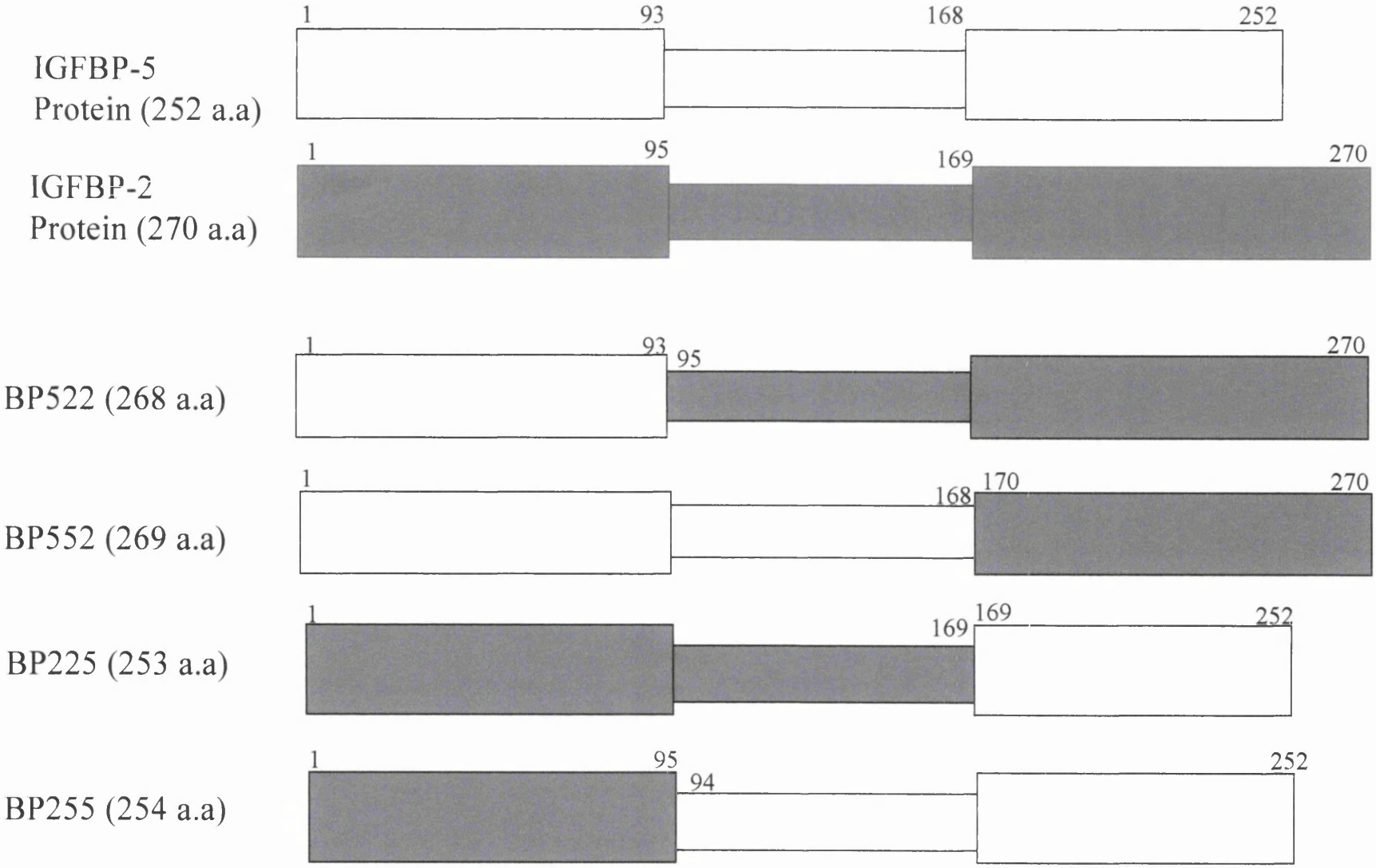
We have made four domain switched chimeric IGFBPs as described in chapter II, and have compared the IGF binding kinetics between wild type and chimeric IGFBPs. The chimeric IGFBPs are named BP552, BP522, BP255, and BP225 to reflect the domains that they contain (Figure IV.1).

IV.2 Results

IV.2.1 Construction of BP552, BP522, BP255, and BP225 chimeric cDNAs

Four chimeric rat IGFBP cDNAs were constructed using mutagenesis to create a unique XhoI site at the junction between exon1 and 2, and exon 2 and 3, in both rat IGFBP-5 and –2. Following digestion with XhoI, the domains were switched by "cutting and pasting" (described in more detail in chapter II). The introduced XhoI sites were designed carefully, so that the open reading frames would be maintained downstream following cutting and pasting. The rat IGFBP-2 cDNA (977bp) consists of 65 bp 5' and 3' untranslated sequence (Figure III.3, see page 96), 393bp exon1, 210bp exon2, 148bp exon3, and 161bp exon4. Exon1 and 2 encode the N- and central-domains respectively, whereas exon3 and 4 encode the C-terminal domain. The rat IGFBP-5 (964 bp) cDNA consists of 95bp 5' and 3' untranslated sequences (Figure III.3, see page 96), 390bp exon1, 228bp exon2, 120bp exon3, and 131bp exon4. The exons encode the same domains described for the rat IGFBP-2 cDNA. Figureure IV.2 shows the results of XhoI restriction analyses of pFastBac1 clones containing wild type IGFBP-5 and -2 and the initial XhoI containing IGFBP-5 and -2 mutants termed BP5/XhoI and BP2/XhoI (XhoI site between exons 1 and 2 of IGFBP-5 and -2 respectively) and XhoI/BP5 and XhoI/BP2 (XhoI site between exons 2 and 3 of IGFBP-5 and -2 respectively). Although there are no XhoI sites within the wild type rat IGFBP-5 and -2 cDNA sequences, there is one XhoI restriction site within the pFastBac1 plasmid. Therefore, pFastBac1 WTIGFBP-5 and WTIGFBP-2 are linearised following digestion with XhoI to create 5739 and 5747 bp fragments respectively. After successful mutagenesis to create BP5/XhoI and XhoI/BP5, the new XhoI restriction sites within the IGFBP-5 sequence results in the release of 624 and 396 bp

Fig. IV.1 Schematic diagram of IGFBP-2 and -5 hybrid proteins



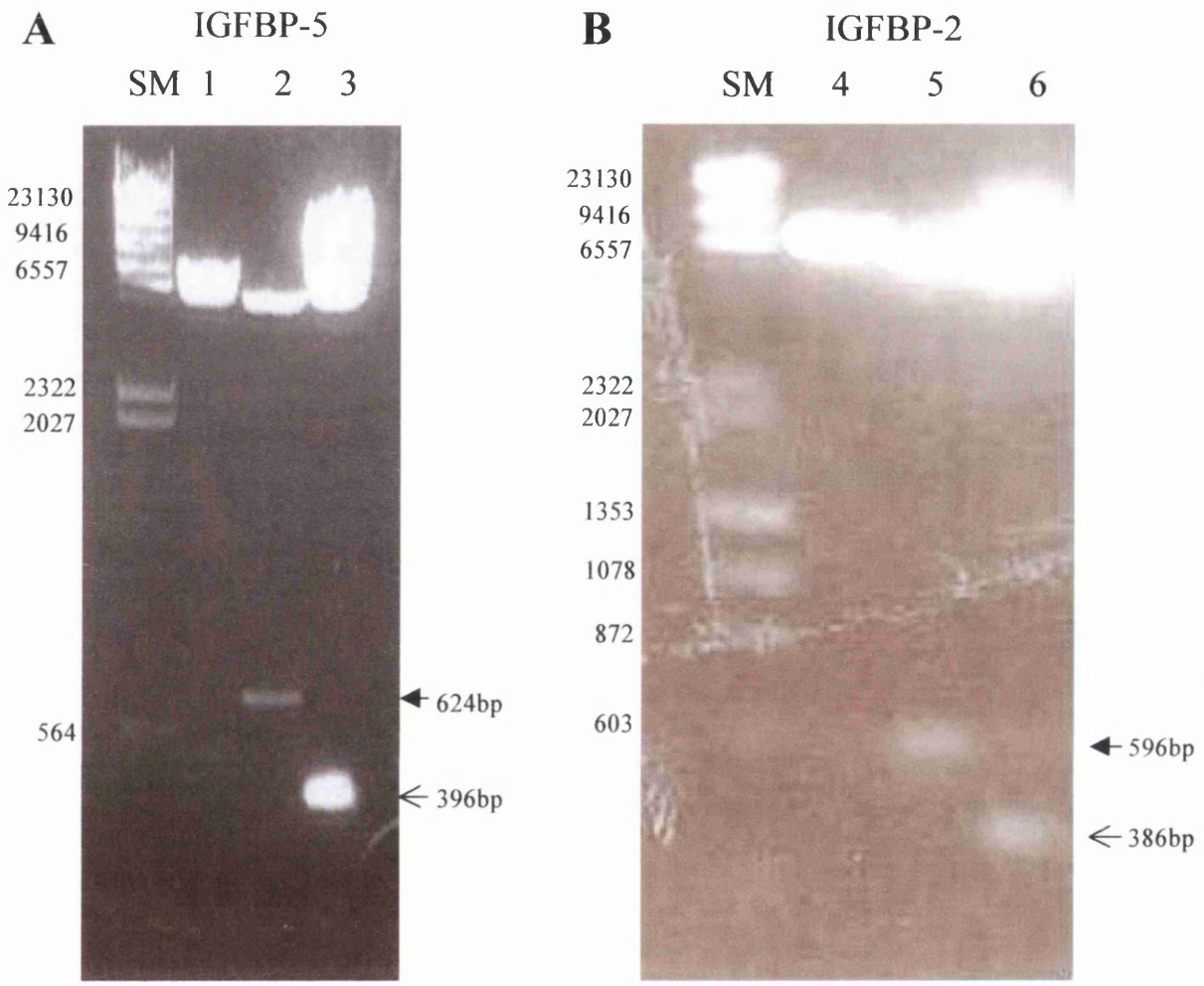


Fig IV.2 (**A**) XhoI digests of pFastBac1 wtIGFBP-5 and mutant cDNAs, lane 1: wild type IGFBP-5, lane2: BP5/XhoI, Lane3: XhoI/BP5. (**B**) XhoI digests of pFastBac1 wtIGFBP-2 and mutant cDNAs, lane4: wild type IGFBP-2, lane5: BP2/XhoI, Lane6: XhoI/BP2. SM represents λ-hindIII (A), λ-HindIII /ϕχ174 (B) DNA size markers, and arrows indicate newly created cleavage Bands.

fragments respectively (Figure IV.2A). Digestion of BP2/XhoI and XhoI/BP2 results in the release of 596 and 386 bp fragments respectively (Figure IV.2B). After "cutting and pasting" the various domains of rat IGFBP-2 and –5, the intermediate chimeric mutants still contain an XhoI site. Figure IV.3A shows the results of an XhoI restriction enzyme digestion of these clones, where BP552, BP225, BP522, BP255 release 386, 396, 596 and 624bp fragments respectively.

To restore the wild-type IGFBP-5 and –2 sequences at the various junctions in the chimeric cDNAs, and thereby remove the XhoI sites, a second round of mutagenesis was performed using the oligonucleotides described in chapter II. These final chimeric IGFBPs clones, containing completely wild type sequence, were also digested with XhoI. The results are shown in Figure IV.3B, where the BP552, BP225, BP522, BP255 are now only linearised at the XhoI site contained within the pFastBac1 plasmid. To confirm this restriction analyses, all four chimeric cDNAs (10μg) were sequenced by MWG biotech (Milton Keynes, UK).

IV.2.2 Expression and primary analysis of non-purified chimeric proteins

The four chimeric proteins were expressed using the baculovirus expression system. To detect the presence of hybrid mutant proteins in the insect cell supernatants, equal volumes of the conditioned media were collected following an infection time-course and western immunoblotted. Three of the chimeric proteins were readily detected by either anti-IGFBP-5 and/or IGFBP-2 antibody in the unconcentrated culture medium as a major band of approximately 30~34kDa (data not shown). However, BP225 was not detected in the culture medium in ten separate attempts to express this chimeric protein. Nevertheless, we decided to concentrate the culture medium from a BP225 infection ten-fold, along with the other three chimeras, using a centricon ultrafree-20 column (Millipore, UK) as described previously.

Following concentration, the proteins were subject to electrophoresis on a 12.5% polyacrylamide protein gel under non-reducing conditions and the gel stained with coomasie blue in an attempt to determine the different ratios of protein concentrations relative to WTIGFBP-5 and -2 (Figure IV.4). Western immunoblotting was not considered appropriate for determining these ratios, since the chimeric proteins will have varying immunogenic responses to the anti-IGFBP-5 and -2

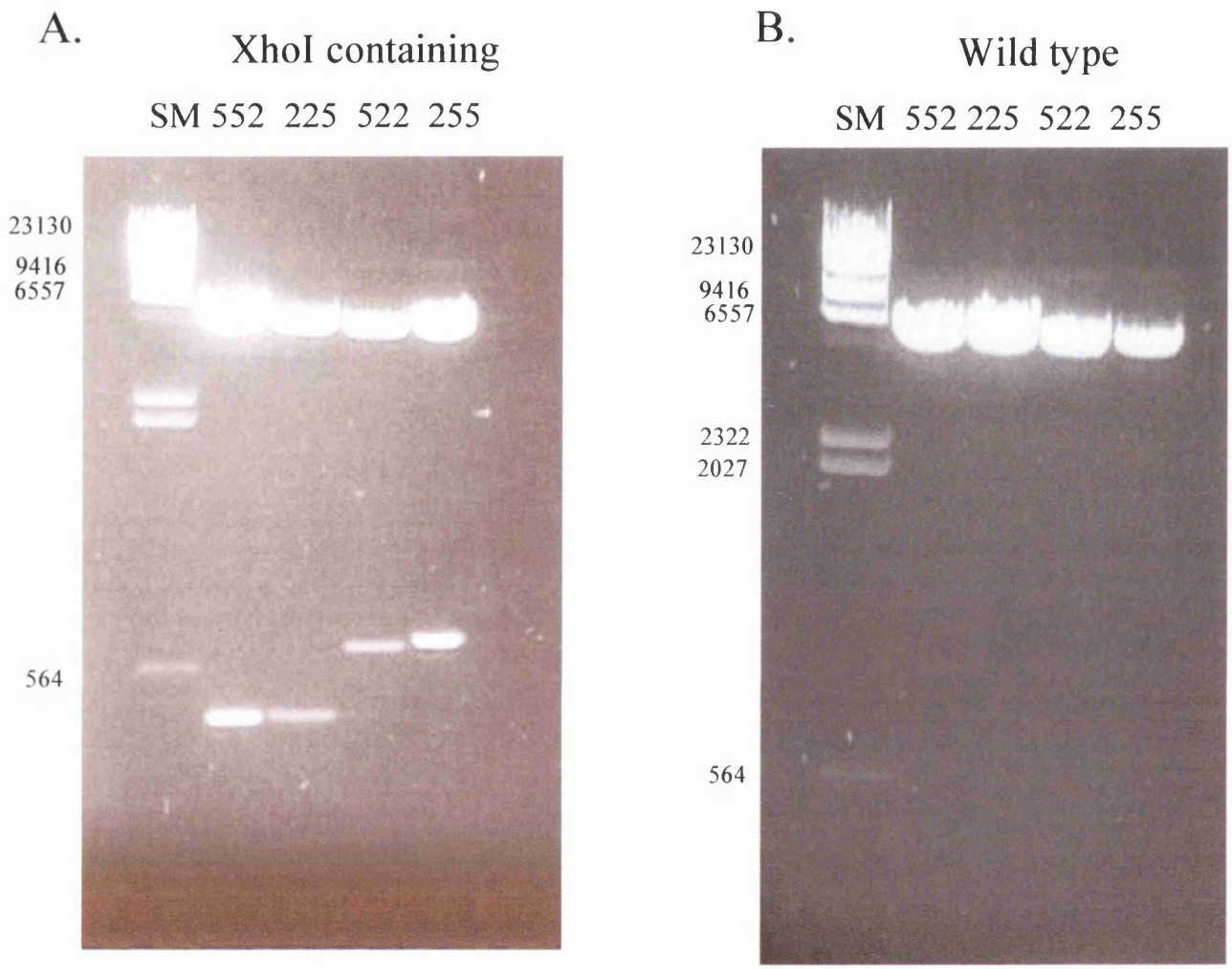


Fig IV.3 (A) XhoI digests of XhoI containing pFastBac1 IGFBP-5 and -2 chimeric cDNAs. (B) XhoI digest of wild type chimeric IGFBP-5 and -2 cDNAs. SM represents a λ-HindIII DNA size markers.

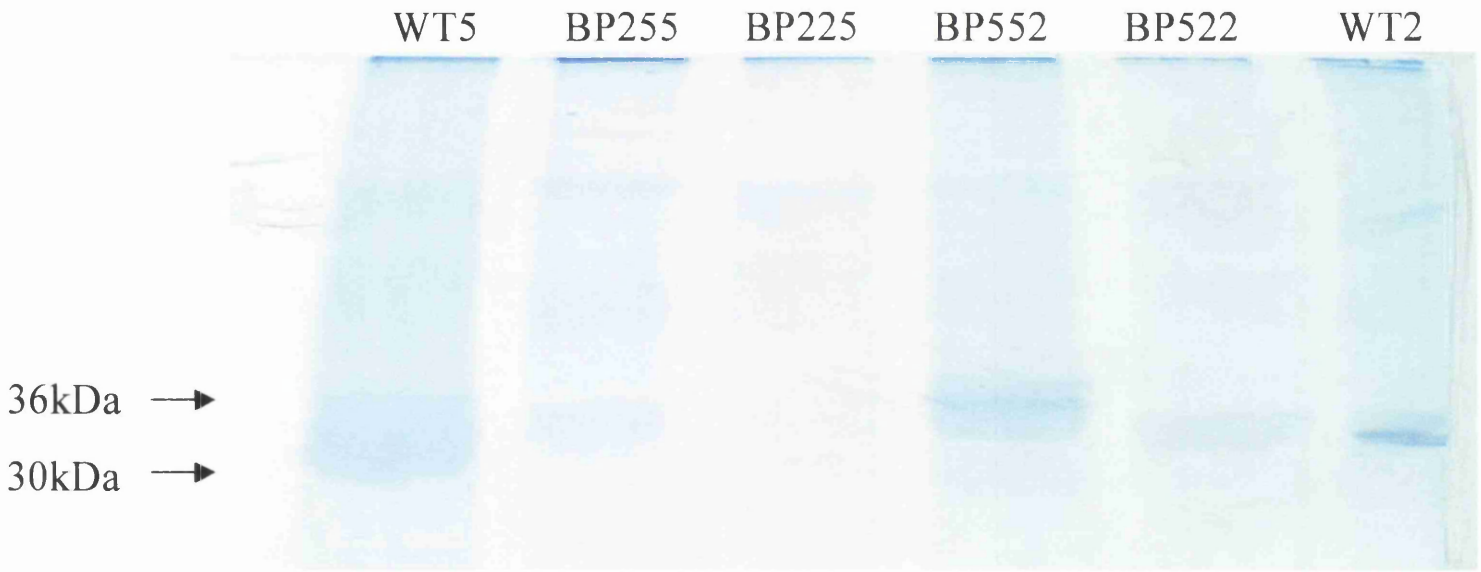


Fig IV.4 Protein quantify using Coomasie blue staining method described in chapter II. Equal volumes of concentrated WT5, BP255, BP225, BP552, BP522, and WT2 culture medium were loaded on the 12% SDS-phase gel under non reducing conditions. Stained gel were applied to densitometric analysis to compare the protein concentration.

antibodies. No protein species of the correct size (30~34kDa) could be detected in the concentrated BP225 culture medium (Figure IV.4). Densitometry was carried out on this gel to get an estimate of protein concentration for subsequent western ligand and immunoblot analysis. Based on this, an attempt was made to load equal amounts of WTIGFBP-5, WTIGFBP-2, BP552, BP522 and BP255 protein, whereas the maximum well volume was loaded for BP225 (approx 25µl). The results of western ligand blotting and immunoblotting with these protein samples are shown in Figure IV.5 (this experiment was repeated seven times with similar results in each instance). From the western immunoblot (IV.5B), the first point of note is that the anti-rat IGFBP-5 and anti-rat IGFBP-2 antisera only recognise the binding protein to which they were raised and do not cross react with the other binding protein. It is also clear that the polyclonal anti-rat IGFBP-5 antisera has epitopes in the N-terminal domain of IGFBP-5, (as BP522 is recognised), and other epitopes elsewhere in the central and/or C-terminal domain (as BP255 is recognised). From our data we can only be sure that the anti-rat IGFBP-2 antisera has epitopes in the C-terminal domain of IGFBP-2 (as BP552 is recognised). For BP225 our data suggests very weak recognition by the IGFBP-2 antisera, although there is only a smear with the IGFBP-5 antisera. From the IGF ligand blots (IV.5B), it would appear that BP552 and BP522 are comparable to the wild type binding proteins in their binding affinities for both IGF-I and -II, whereas BP255 does not appear to bind IGF-I, and only binds IGF-II very weakly. The binding of BP225 to IGFs is inconclusive, as the evidence would strongly suggest that this protein is not properly expressed. Based on this evidence, BP225 was excluded from further analyses.

The IGF binding properties of the remaining three chimeric proteins were also analysed in ^{125}I-IGF-I or ^{125}I-IGF-II solution phase binding assays, and the results are shown in Figure IV.6. These results support the ligand blot data, where BP552 and BP522 have affinities for binding IGFs that are equivalent to the wild type proteins, whereas the affinity of BP255 for binding IGFs is greatly compromised. This experiment was repeated four times, with similar results in each instance.

Based on the above data from ligand blot and solution phase data, it was decided to purify BP552 and BP522 by IGF affinity chromatography, so that the IGF binding characteristics of these two chimeric proteins could also be analysed in the BiaCore system.

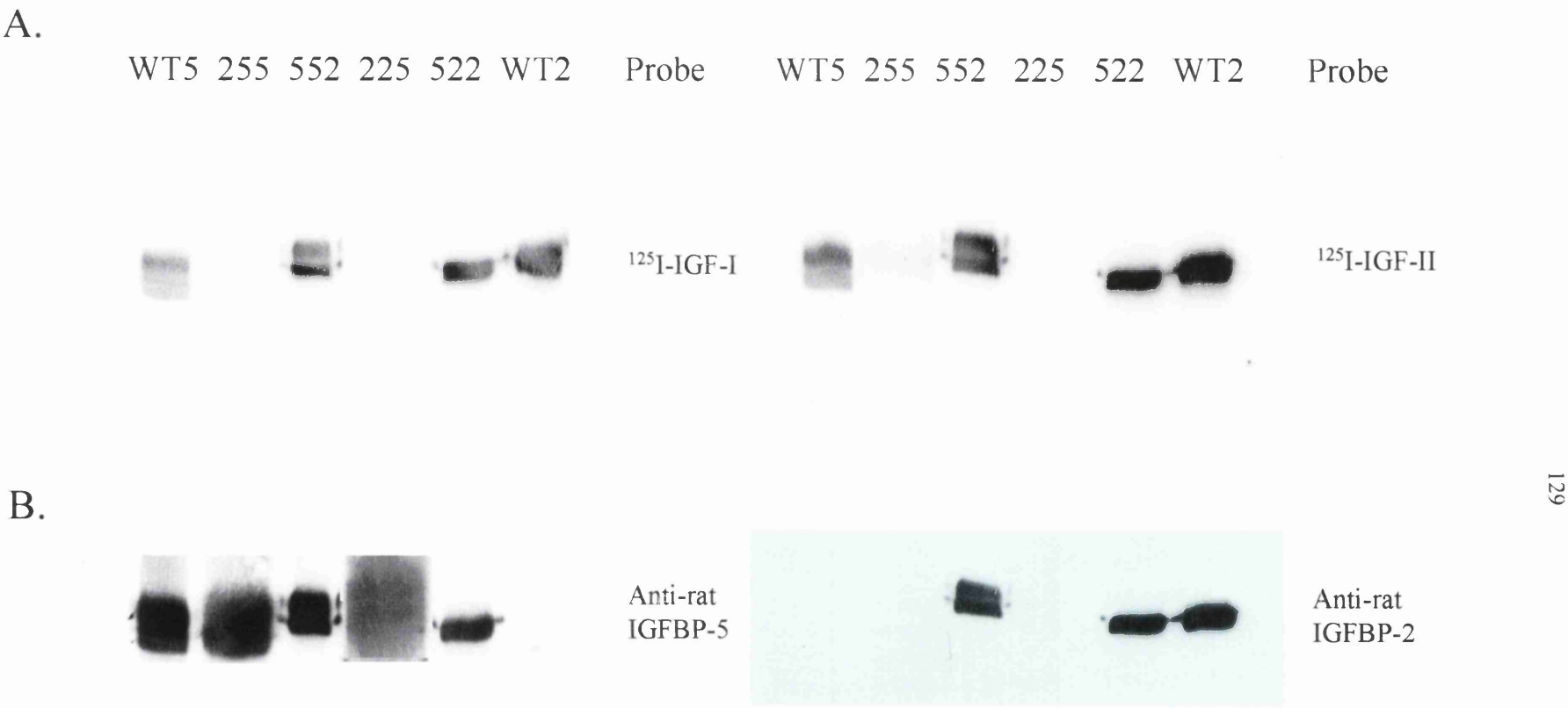
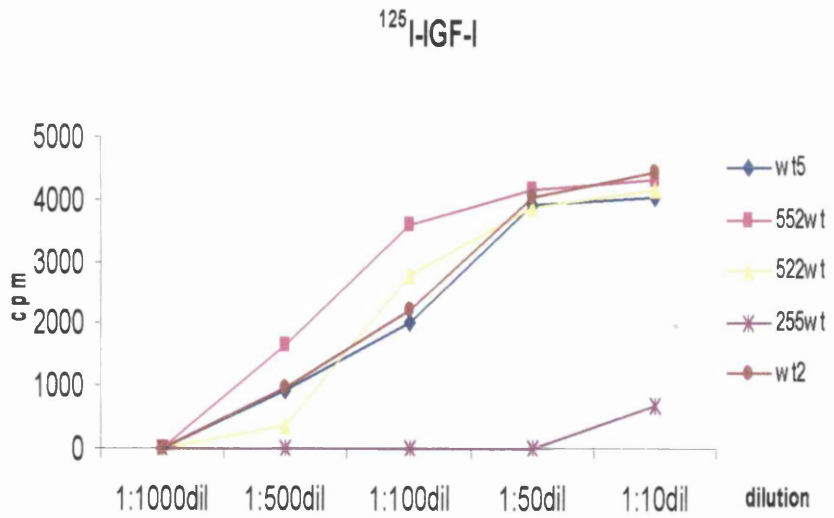


Fig IV. 5 (A) ^{125}I-IGF-I and -II ligand blots of concentrated wt IGFBP-5, BP552, BP522, BP225, BP255 and wtIGFBP-2 culture medium. Ligand blotting was performed as described in chapter II. (B) Protein loading levels were monitored by probing the same blots with either polyclonal anti-rat IGFBP-5 or anti-rat IGFBP-2 antibodies (lower panel) after development of ligand lots.

A.



B.

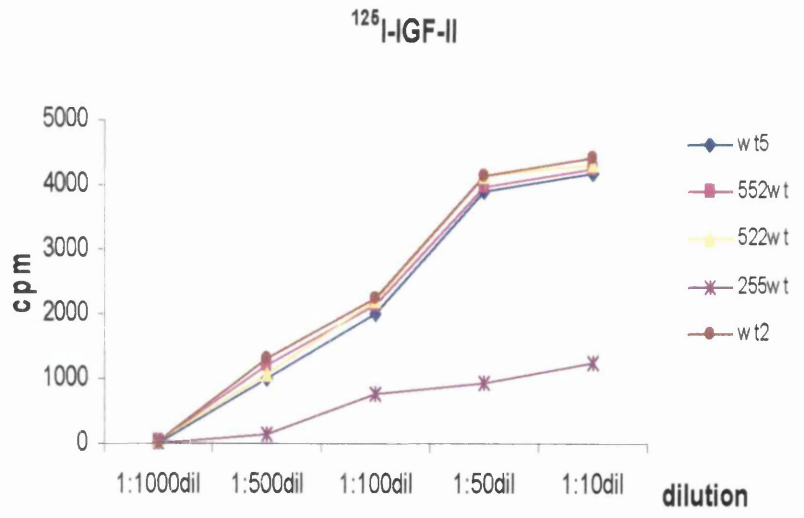


Fig IV.6 Solution phase IGF binding assays. The binding curves for unpurified, concentrated insect cell culture medium containing wild type IGFBP-5 and -2, BP552, BP522 and BP255 binding to (A) ^{125}I-IGF-I or (B) ^{125}I -IGF-II are shown.

IV.2.3 Analysis of purified chimeric proteins

Both BP552 and BP522 were applied to an IGF-II affinity column following the method described in chapter II. Subsequently, the concentration of the purified proteins was determined by Bradford assay (BP552 and BP522 were 45 and 26ug/ml respectively). The purified proteins were first analysed by western immuno blotting using the anti-IGFBP-2 and -5 antisera described above (Figure IV.7) (these experiments were repeated three times, with similar results obtained on each occasion).

Solution phase IGF binding assays were also carried out on the purified BP552 and BP522 chimeras and the data were applied to Scatchard transformation (Scatchard, 1949) to generate linear regression binding curves (Figure IV.8) (this experiment was repeated four times, with similar results in each instance). From this analyses, IGFBP-5, BP552, BP522, and IGFBP-2 proteins were calculated to have Kd values of 0.061, 0.62, 0.036 and 0.0.038nM respectively for binding to IGF-I (Figure IV.8.A), and 0.024, 0.034, 0.028 and 0.021nM respectively for binding to IGF-II (Figure IV.8.B). Therefore, from this experiment it would appear that all four proteins have essentially the similar affinity whether IGF-I or IGF-II is used as ligand. However overall there appears to be an approximately 2-fold higher affinity for IGF-II.

Finally, a BiaCore analysis was undertaken to further investigate the IGF binding properties of BP552 and BP522 (Table IV.1 and Figure IV.9). In Figure IV.9, the individual sensograms are presented for WTIGFBP-5, WTIGFBP-2, BP552 and BP522 binding to both human IGF-I and IGF-II immobilised on the surface of biosensor chips. The binding curves clearly indicate an interaction of all four proteins with both IGF-I and IGF-II. The data are summarised quantitatively in Table IV.1, where we demonstrate K_D values for WTIGFBP-5 protein of 10^{-8} to 10^{-9} M. In this system, both BP552 and BP522 display wild type like binding for both IGFs, with their affinity constants comparable to that for WTIGFBP-2 and -5. Interestingly, the data for WTIGFBP-2 demonstrates that this binding protein not only has a 20-fold higher affinity than WTIGFBP-5 for binding IGF-I, but that it also has a 5-fold higher affinity for IGF-I than IGF-II.

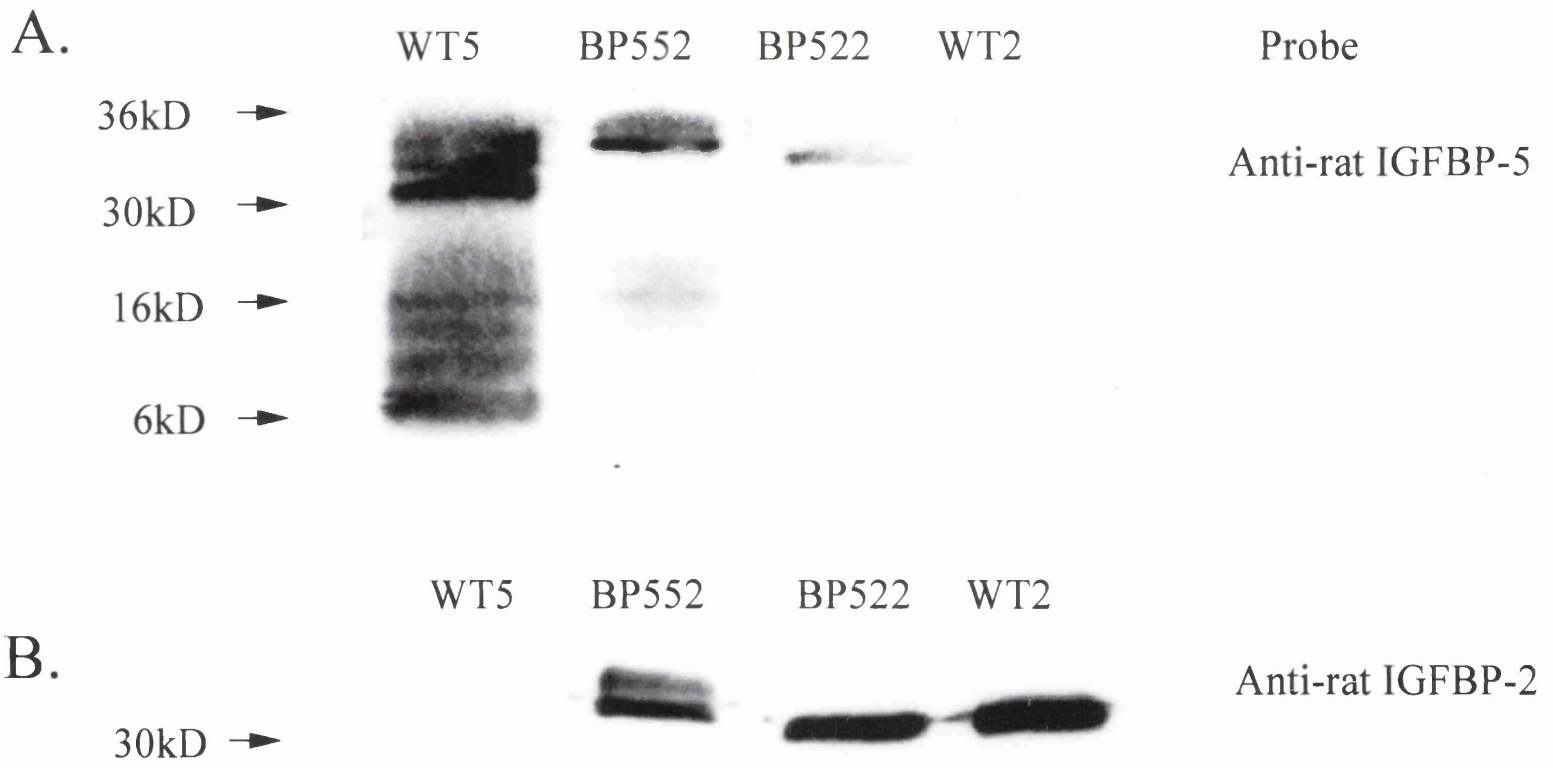


Fig IV.7 Western immunoblots of purified wtIGFBP-5 (WT5), BP552, BP522, and wtIGFBP-2 (WT2). Proteins were loaded on 12% polyacrylamide gels and the blots were probed with either a polyclonal anti-rat IGFBP-5 (**A**) or anti-rat IGFBP-2 antiserum (**B**).

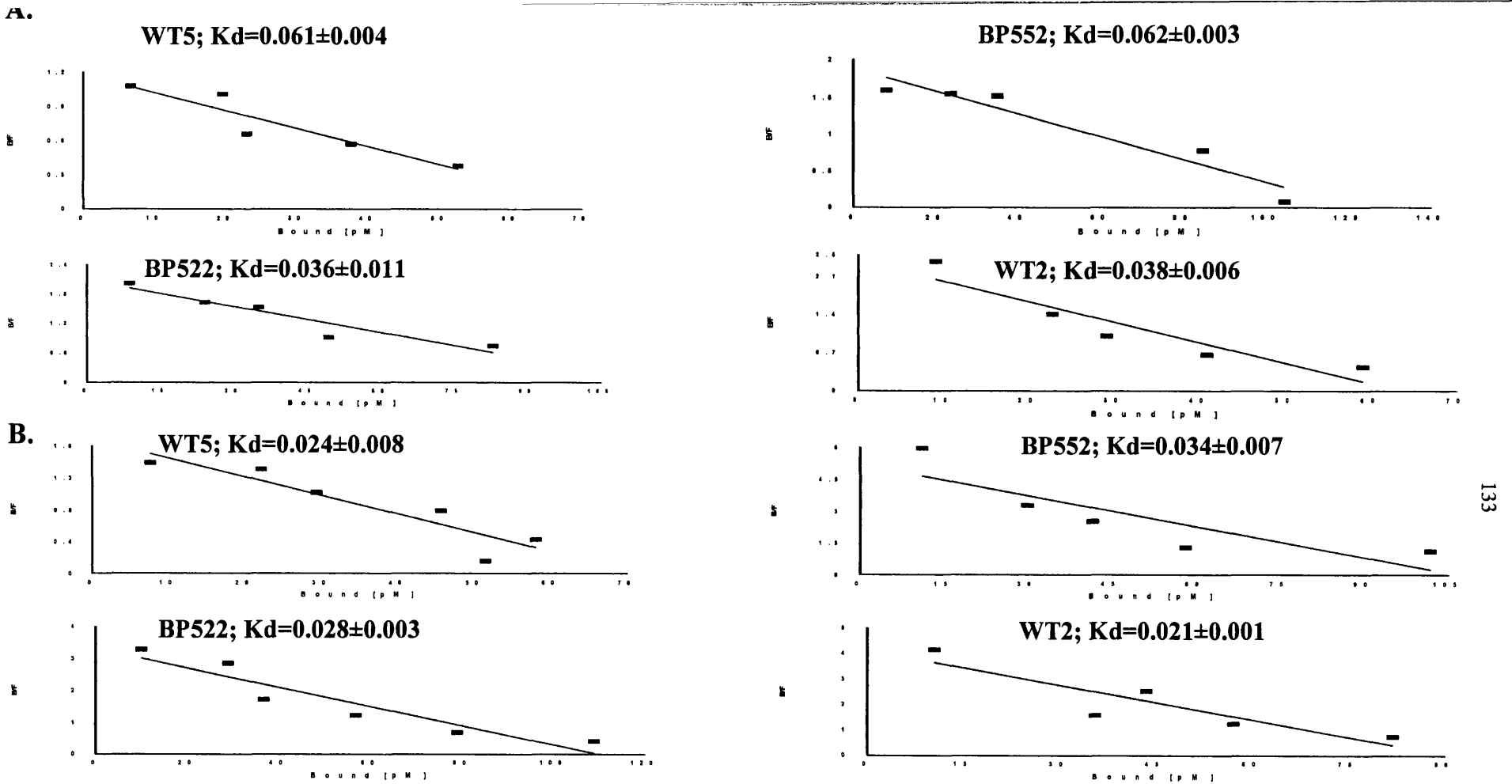


Fig IV.8 Solution-phase assay of IGFBP activity. wtIGFBP-5, wtIGFBP -2, BP552 and BP522 were analysed by solution-phase binding assay as described in chapter II. Scatchard analysis was derived after the determination of IGFBP binding to (A) ^{125}I-IGF-I or (B) ^{125}I-IGF-II in the presence of increasing concentrations of unlabelled IGF-I or -II. In both instances, regression lines are drawn through the data obtained by Scatchard analysis. B/F, bound/free. Kd=Mean (Kd) ± Standard Error. The mean Kd values in this figure were derived from 3 or 4 separate experiments with the individual clones.

Table IV.1 BIA core analysis

Protein	IGF-I			IGF-II		
	$K_{ON}(Ms^{-1})$	K_{OFF} (s^{-1})	K_D (M^{-1})	$K_{ON}(Ms^{-1})$	K_{OFF} (s^{-1})	K_D (M^{-1})
IGFBP-5	6.58×10^{4}	5.06×10^{-3}	7.68×10^{-8}	4.53×10^{5}	2.93×10^{-3}	$6.47 \times 10^{-}$
BP552	1.03×10^{5}	1.18×10^{-3}	1.14×10^{-8}	6.30×10^{4}	6.04×10^{-4}	$9.58 \times 10^{-}$
BP522	2.64×10^{4}	7.82×10^{-4}	2.97×10^{-8}	1.84×10^{4}	1.42×10^{-3}	$7.87 \times 10^{-}$
IGFBP-2	1.11×10^{5}	4.09×10^{-4}	3.69×10^{-9}	1.74×10^{4}	3.19×10^{-4}	$1.83 \times 10^{-}$

Biacore analysis of kinetic constants for WTIGFBP-5, BP552, BP522, and WTIGFBP-2 binding to immobilized IGF-I and –II. General curve fitting was performed for 8.75, 4.38, 2.19 and 1.04 nM of analyte – using software provided by Biacore.

K_D is derived as K_{OFF} / K_{ON}.

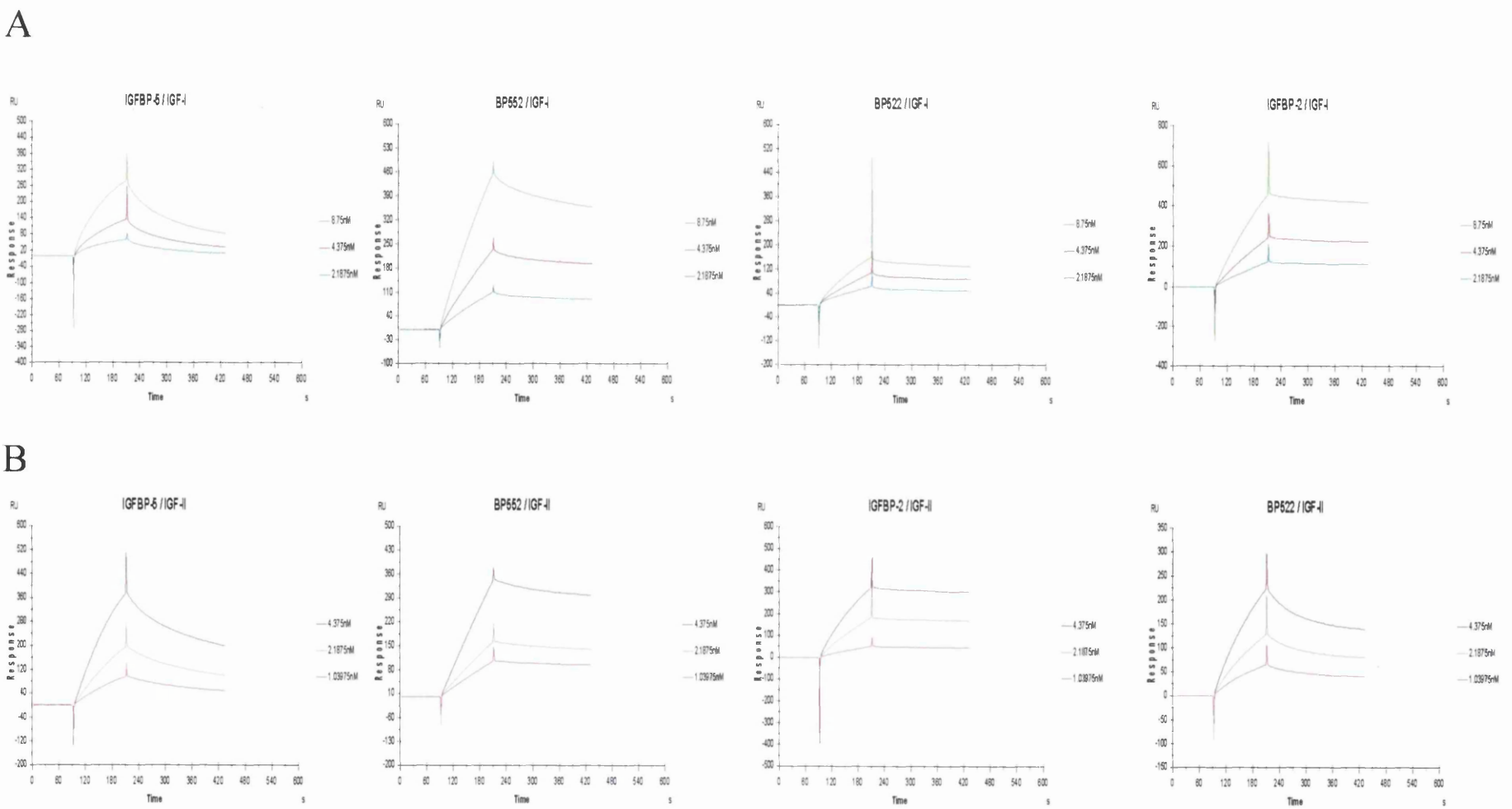


Fig IV.9 Individual sensograms for wtIGFBP-5, wtIGFBP -2, BP552 and BP522 binding to both human IGF-I (A) and IGF-II (B). The sensograms show real time K_{ON} and K_{OFF} values with three different concentrations of IGFBPs (top, middle and bottom line corresponding to 8.75nM, 4.38nM and 2.19nM respectively for IGF-I; top, middle and bottom line corresponding to 4.38nM, 2.19nM and 1.04nM respectively for IGF-II).

IV. 3 Discussion

We have attempted to express four chimeric IGFBP proteins made from switching domains between rat IGFBP-5 and -2 in the baculovirus/insect cell system. While we are able to detect expression of three of these proteins, we were consistently unable to detect expression of one of the chimeras, BP225. One possible explanation for this is that this recombinant protein cannot be properly folded by the insect cells and, as a result, may be susceptible to proteolysis. In support of this, western immuno analysis using the anti-rat IGFBP-5 antiserum only detected a smear for BP225, which is suggestive of proteolysis (FigureIV.5B). BP552, BP522 and BP255 were all detectable by both Coomassie blue staining and western immunoblotting. Our results indicate that the anti-rat IGFBP-5 and anti-rat IGFBP-2 antisera do not cross react with the other binding protein, so that the recognition of BP552 and BP522 by both antisera (Figures IV.5B and IV.7) confirms their identity as chimeric proteins made up from domains from both IGFBP-5 and -2. BP255, on the other hand, was only recognised by the anti-rat IGFBP-5, but this could be explained by the anti-rat IGFBP-2 antiserum lacking epitopes in the N-terminal domain of IGFBP-2.

As discussed earlier, IGFBP-5 normally appears as a doublet when expressed from insect cells, whereas IGFBP-2 is a single band. We have previously postulated that this may be the result of inefficient cleavage of the signal peptide from IGFBP-5. However, this does not explain our results for BP522, which appears as a single band in our Westerns, whereas BP552 still appears as a doublet (Figures IV.5B and IV.7). This strongly suggests that the double band is due to some effect of the central domain of IGFBP-5. Others have previously reported that IGFBP-5 undergoes post-translational modifcations (O-linked glycosylation and phosphorylation) specifically in the central domain (Conover & Kiefer 1993, Coverley & Baxter 1997). Therefore, one obvious explanation for the IGFBP-5 doublet is altered post-translational modification of the recombinant protein by the insect cells. Post-translational effects in the central domain could also explain why BP552 migrates at a slightly higher molecular weight than WTIGFBP-2 and BP522 (Figures IV.5B and IV.7), even though these three proteins have almost exactly the same number of amino acids in their sequence (BP552, WTIGFBP-2 and BP522 contain 269, 270 and 268 amino acids respectively, whereas WTIGFBP-5 has only 252). Finally, we also observe a greater number of proteolytic fragments from WTIGFBP-5 compared to BP552 using the anti-rat IGFBP-5 antiserum (Figure IV.7A). This

is initially surprising, as most of the proteolytic sites in IGFBP-5 are believed to be within the central domain (discussed in more detail in chapter V). However, the apparent greater number of fragments from the WTIGFBP-5 protein could also be explained by the antiserum detecting fragments containing sequences from the C-terminal domain of IGFBP-5.

Our solution phase IGF binding analyses for WTIGFBP-5 and -2 did not strictly agree with the results of Keifer *et al*,. (1992), in which they observed several fold higher affinities for IGFBP-5 binding to both IGF-I and -II, compared to IGFBP-2. In our work, we found that WTIGFBP-5 and -2 had very similar affinities irrespective of ligand, with both binding proteins having an approximately 2-fold higher affinity for IGF-II (Figure IV.8). These differences could possibly be explained by the fact that Kiefer *et al*., (1992) studied the interaction of human IGFBPs, either produced recombinantly or derived from human biological sources, with human IGFs, whereas we employed a heterologous system with rat IGFBPs and human IGFs. Furthermore, although our BiaCore study demonstrated K_D values for WTIGFBP-5 and -2 of the same order of magnitude, IGFBP-2 appeared to show a higher affinity for IGF-I (5-fold) than IGF-II, whereas IGFBP-5 maintained a 10-fold greater affinity for IGF-II than IGF-I. Although the general consensus in the literature is that both IGFBP-5 and -2 exhibit a higher affinity for IGF-II than IGF-I, it is important to remember that almost all affinity studies reported to date have employed solution phase binding analyses. The BiaCore system is becoming increasingly recognised as providing more detailed information on molecular interactions, and it may be that our real time kinetics data for IGFBP-2 binding to IGFs reflects a more accurate picture of the ligand preference of this binding protein.

More important in the context of this study are the binding affinities for the chimeric proteins. First, BP225 had to be excluded from this analysis, as it would appear that the insect cells were unable to properly express this protein. As discussed above, this could be the result of improper folding of the recombinant protein, which, in turn, makes BP225 susceptible to proteolysis. On the other hand, although we were able to detect expression of BP255 by western immunoblot (Figure IV.5B), we were unable to detect any binding of this chimera to IGF-I by either solution phase assay (Figure IV.6), or ligand blotting (Figure IV.5A), and only very weak interaction with IGF-II. It is very difficult to draw any firm conclusions about the IGF binding

properties of BP255. One obvious, and potentially very interesting, conclusion would be that the N-terminal domain of IGFBP-2 is simply unable to co-operate with the C-terminal domain of IGFBP-5 in order to bind IGFs with high affinity. However, as with BP225, we cannot rule out the possibility that BP255 is incorrectly folded, (although it would not appear to be proteolysed as it retains immunogenicity for the anti-rat IGFBP-5 antiserum). The reasons why BP225 and BP255 may be more susceptible to folding problems than BP552 and BP522 remain unclear. The N- and central domains of IGFBP-5 and -2 are very similar in size, although the C-terminal domain of IGFBP-2 is significantly larger. Moreover, others have identified the disulphide bonding pattern in the N-terminal domain of IGFBP-5 (Kalus *et al*, 1998; Neuman et al, 1999) and the C-terminal domain of IGFBP-2 (Forbes *et al*, 1998), and consequently have established that there are no inter-domain disulphide bonds in either of these two binding proteins. Therefore, the IGFBP model of two highly structured terminal domains being held apart by a flexible central domain should also hold true for both IGFBP-5 and -2. Further elucidation of the IGF binding properties of BP255 would require careful structural analysis, such as nuclear magnetic resonance (NMR) and/or circular dichroism (CD) spectroscopy.

The two remaining chimeric proteins, BP552 and BP522, have comparable affinities with the native IGFBP molecules from which they were derived for binding IGF-I and IGF-II, as measured by our solution phase and biosensor experiments. We conclude from this finding that these hybrid proteins do not have major structural alterations induced in their N- and C-terminal domains, as this would have been expected to cause a marked decrease in their affinity for ligands. Although the affinity constants of WTIGFBP-5, WTIGFBP-2, BP552 and BP522 were all similar in solution phase assay (Figure IV.8), the BiaCore analysis highlighted the differences in binding affinities. For IGF-I binding, K_D values for the chimeric proteins fall within a range between the wild type proteins: WTIGFBP-2 > BP552 > BP522 > WTIGFBP-5, whereas for IGF-II the order was WTIGFBP-5 > BP552 > WTIGFBP-2 > BP522 (Table IV.1). This demonstrates that the N-terminal domain of IGFBP-5 is able to co-operate with the C-terminal domain of IGFBP-2 to obtain high affinity IGF binding, irrespective of the central domain that is present.

Although the main objective of this study was to identify the particular contribution of the N- and C-terminal domains in binding IGFs, it is very difficult to say from the data above whether a

specific domain has a specific ligand binding preference. This is largely because we lack data for IGF binding to two of our chimeras (BP255 and BP225), but also that the affinities of the WTIGFBP proteins in our studies were markedly different from that previously reported in the literature. Based on previous proteolytic analysis of IGFBP-5, where it was found that a 23kD N-terminal fragment is able to bind weakly to IGF-II (Imai *et al*, 1997; Standker *et al*, 1998), while a 13.5kD C-terminal fragment binds IGF-I (Standker *et al*, 1998) it could simply be concluded that the sequences responsible for IGF-II and -I preference are contained within the N- and C-terminal domains respectively. Our data from the chimeric protein study can neither confirm or refute this hypothesis. In summary, we have made chimeric proteins containing domains from rat IGFBP-5 and -2 and have shown that the N-terminal domain of IGFBP-5 can co-operate with the C-terminal domain of IGFBP-2 to obtain high affinity IGF binding, irrespective of which central domain is present, whereas preliminary evidence suggests that the N-terminal domain of IGFBP-2 cannot co-operate with the C-terminal domain of IGFBP-5 for high affinity IGF binding.

Chapter V. A cryptic heparin-binding site in the central domain of IGFBP-5

V.I. Introduction

As discussed in chapter I.3.4, IGFBP-5 has three putative consensus heparin binding motifs, one in the basic region within the C-terminal domain, YKRKQCKP (205-212), and two overlapping in its central domain, VKKDRRKKL (132-140) (Hodgkinson *et al.* 1997). The central domain heparin binding motifs have previously been ruled out by others as functional heparin binding sites based on studies with the full-length IGFBP-5 protein. This included both competitive binding studies with synthetic peptides from these regions (Arai *et al.* 1994b) and amino acid substitutions at these putative heparin binding motifs (Arai *et al.* 1996a), neither which resulted in a change in IGFBP-5 heparin binding properties.

Domain switching to create chimeric IGFBP proteins has previously been adopted by other groups in order to ascertain which domains of IGFBP-3 and IGFBP-5 are involved in binding to a liver derived glycoprotein known as the acid-labile subunit (ALS) (Hashimoto *et al.* 1997, Twigg *et al.* 1998). Using this approach, it was demonstrated that the homologous stretch of basic amino acids in the C-terminal domains of IGFBP-3 (214-232) and IGFBP-5 (201-218) is necessary for ternary complex formation with ALS and IGFs. In addition, it was shown that at high concentrations, the GAGs heparin and heparan sulfate inhibited ALS binding to IGFBP-5 when it was bound to IGF, suggesting overlap of heparin and ALS binding sites in the C-terminal domain (Twigg *et al.* 1998). Further analysis of IGFBP-5/IGFBP-6 chimeric proteins with N-terminal and central domain swaps, and a C-terminally truncated mutant, IGFBP-5^{1-169}, has revealed the existence of a site in the central domain of IGFBP-5 that binds to ALS in the absence of the IGFBP-5 C-terminal domain (Twigg *et al.* 2000). Analogous to the overlap of heparin and ALS binding sites in the C-terminal basic region, these authors speculated that the ALS may bind somewhere within the two overlapping heparin binding sites at 132-140 in the central domain.

In order to assess the contribution of the C-terminal and central heparin binding sites to the function of IGFBP-5, we have employed two of our chimeric IGFBP proteins, BP552 and BP522,

made by switching domains between rat IGFBP-5 and rat IGFBP-2 (discussed in detail in chapter IV; Figure IV.1). Using biosensor real time analysis of binding kinetics, IGF binding properties were previously determined for WTIGFBP-5 and -2, BP552, BP522, the C-terminaly truncated BP550 and Hep- (chapter III and IV). In this chapter, Heparin ligand blotting was also used to investigate the heparin binding properties of the above proteins and three further IGFBP-5 mutants, Hep-2, Hep-3 and BP550Hep-, in which the mutations R136A:R137A were made in the central consensus heparin binding site of WTIGFBP-5, Hep- and BP550 respectively (Figure V.1). The implications of our findings for the function of IGFBP-5 with respect to proteolysis and interaction with heparin are discussed, and are published in Song *et al*, (2001).

V.2 Results

V.2.1 Construction of Hep-2, Hep-3, and BP550Hep-

To create Hep-2, Hep-3, and BP550Hep-, site-direct mutagenesis were performed on the wild type IGFBP-5, Hep-, and BP550 cDNAs respectively using the 'Altered sites' system (Promega) as explained in chapter II. The mutagenic oligonucleotide used for creating R136A:R137A also creates a restriction endonuclease cleavage site for TseI. Figure V.2 shows the results of TseI restriction digest of of WTIGFBP-5 and the various mutants. There are seven TseI restriction sites within the IGFBP-5 cDNA sequence, and several more in the pFastBac1 plasmid, which creates the complex restriction pattern shown in the WT track. However, succesful mutagenesis to create Hep-2, Hep-3 and BP550Hep-, inserts an extra TseI site and this produces an extra 310bp restriction fragment (indicated with an arrow in Figure V.2). Based on this data, which initially identified our mutant cDNAs, we confirmed the prescence of the mutations by comercial automated sequencing (MWG biotec, Milton keynes, UK).

Hep-2, Hep-3, and BP550Hep- were expressed and purified as described in chapter II, and protein concentration was subsequently estimated by Bradford assay as described in chapter II.

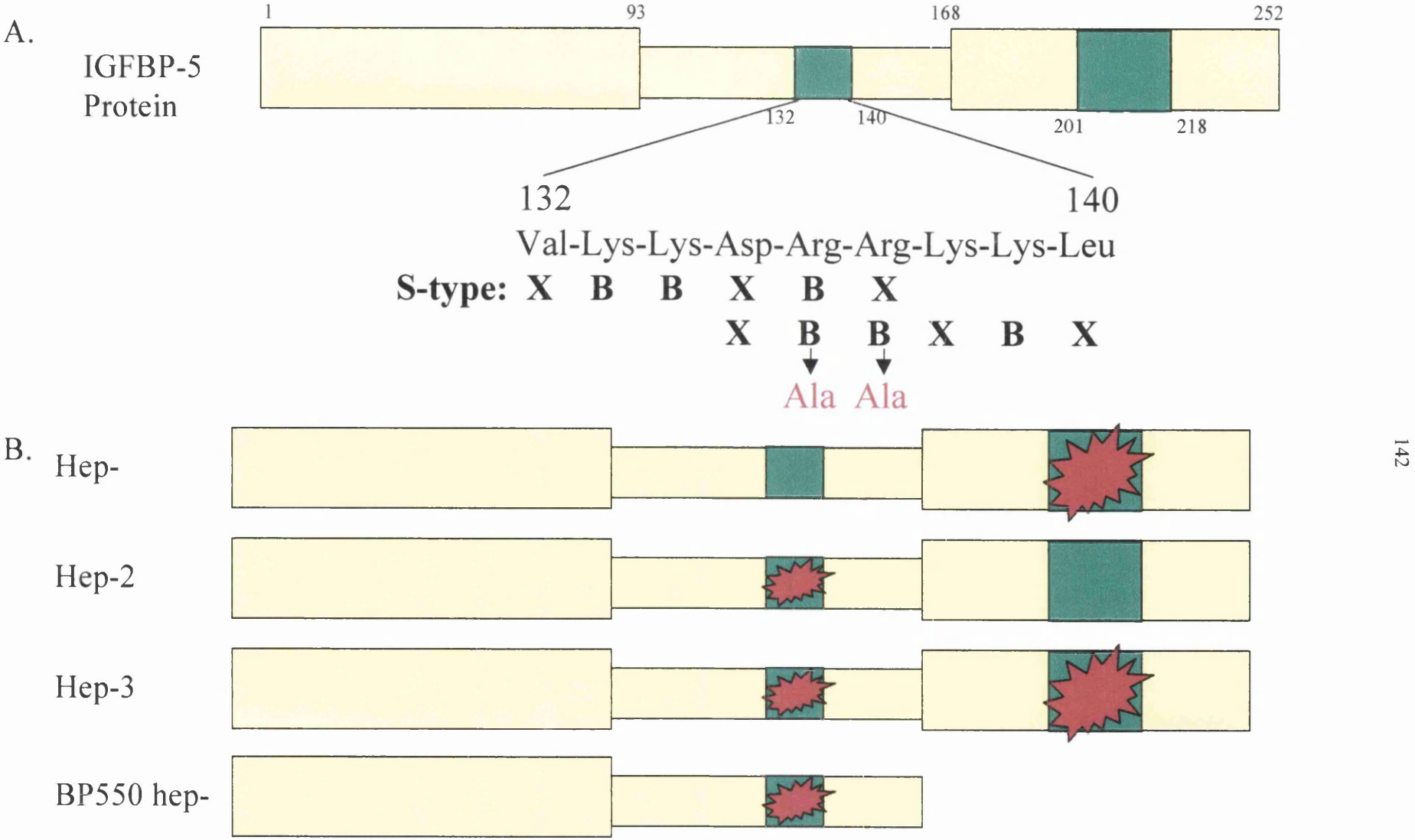


Fig V.1 A. shows a schematic outlining the amino acid substitutions that were made to the central consensus heparin binding sites. B. shows a schematic of the various heparin binding mutants of IGFBP-5 that were employed in this study.

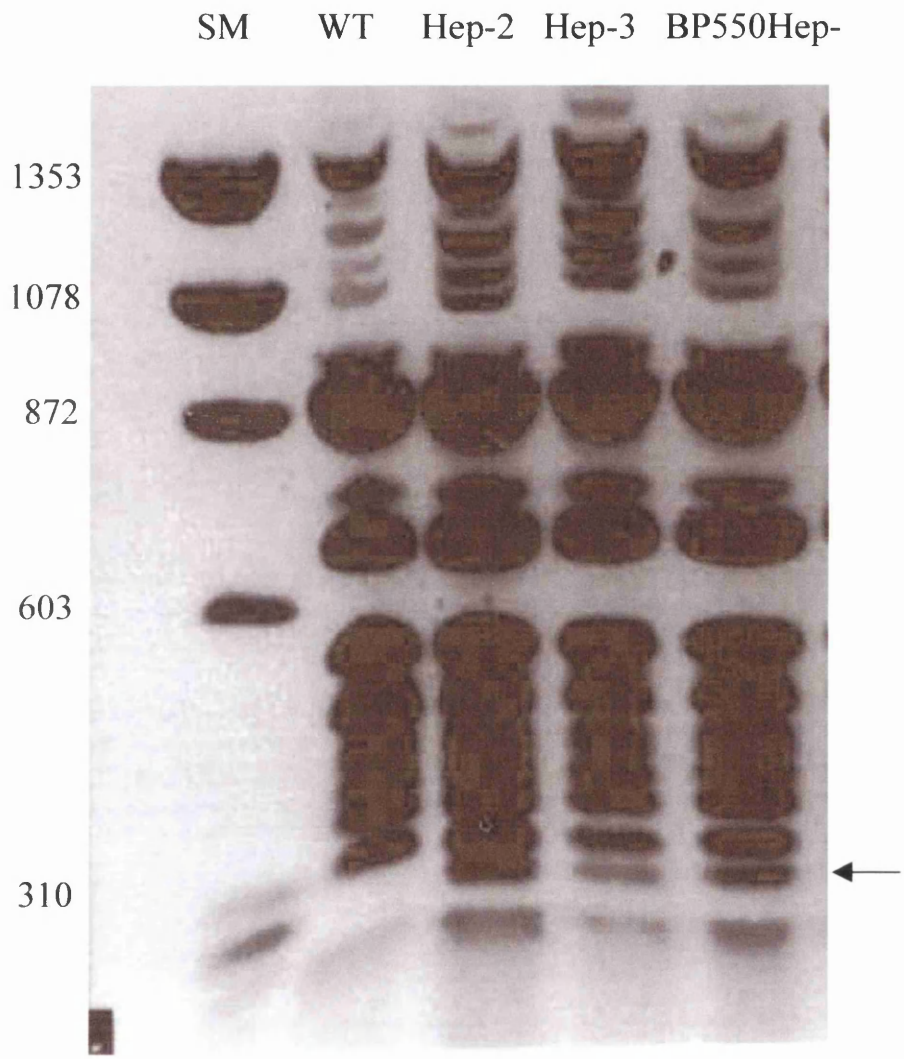


Fig V.2 1% agarose gel of wtIGFBP-5, Hep-2, Hep-3, and BP550Hep- cDNAs, inserted in pFastbac1 plasmid, digested with TseI. SM represents $\phi\chi$ DNA size markers and the sizes are indicated. Arrow indicates newly created restriction fragment.

V.2.2 Heparin-ligand blotting

Previously we have used heparin ligand blotting to study the heparin binding properties of Hep- and other IGFBP-5 mutants (Song *et al.* 2000). This clearly demonstrated that Hep- bound to heparin very poorly in comparison to WTIGFBP-5, and confirmed the identity of some of the important basic residues in the C-terminal domain that are required for heparin binding (Arai *et al.* 1996a, Parker *et al.* 1998). Heparin ligand analysis was initially carried out on BP552, BP522, BP550 and Hep-, and the results are shown in Figure V.3. The great reduction in heparin binding displayed by Hep- again clearly demonstrates that full-length WTIGFBP-5 binds heparin predominantly through its C-terminal domain sequences. However, we found it intriguing that both BP552 and BP550 clearly bound to heparin with equivalent, or even greater, ability than WTIGFBP-5, whereas WTIGFBP-2 and BP522 failed to bind heparin. The absence of heparin binding with the BP522 chimera identifies that the central domain of IGFBP-5 as being involved in the heparin binding activity of BP552 and BP550. This experiment was repeated four times with similar results in each instance.

Based on these findings, we wanted to establish whether this central domain heparin binding activity is accounted for by the consensus GAG-binding sites between residues 132-140. Therefore, we mutated R136A:R137A in WTIGFBP-5, Hep- and BP550 to make the mutants Hep-2, Hep-3 and BP550Hep- respectively (Figure V.1). The effects of these mutations on heparin binding are shown in Figure V.4. It is clear that the R136A:R137A mutations have no appreciable effect in the full-length proteins, as Hep-2 and Hep-3 resemble WTIGFBP-5 and Hep- respectively with regard to heparin binding. However, BP550Hep- fails to bind heparin, which clearly demonstrates that R136 and R137 are essential for BP550 heparin binding. This experiment was repeated three times with similar results in each instance.

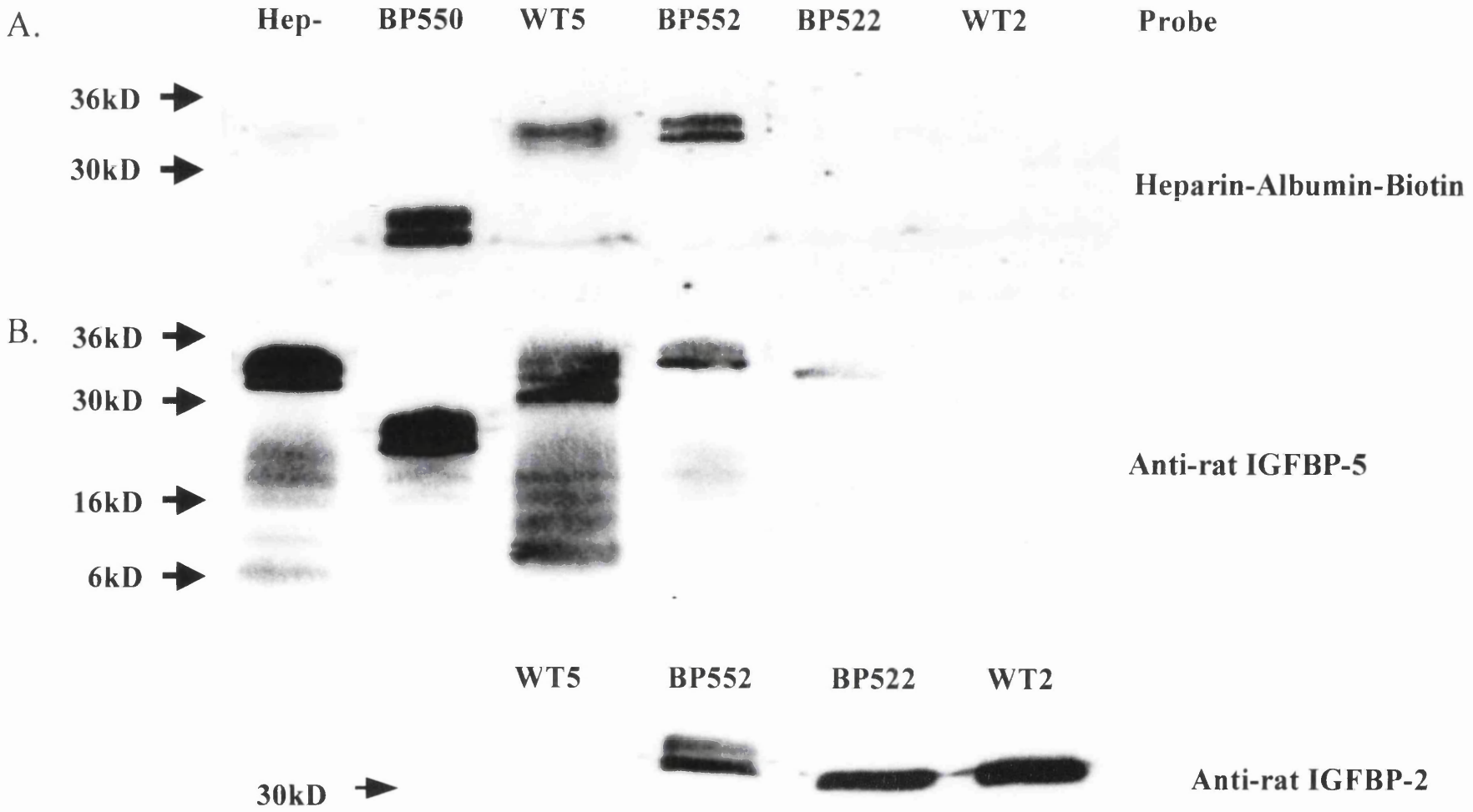


Fig V.3 Heparin ligand blot of Hep-, BP550, wtIGFBP-5, BP552, BP522, and wtIGFBP-2. Proteins were loaded on 15% polyacrylamide gels and run under non-reducing conditions. A. shows the results of Heparin ligand analysis, ligand reactivity was tested with heparin-albumin-biotin and streptavidin-peroxidase. Below in B. are duplicate blots used to monitor protein loading using the anti-rat IGFBP-5 or anti-rat IGFBP-2. This experiment was repeated four times with similar results in each instance.

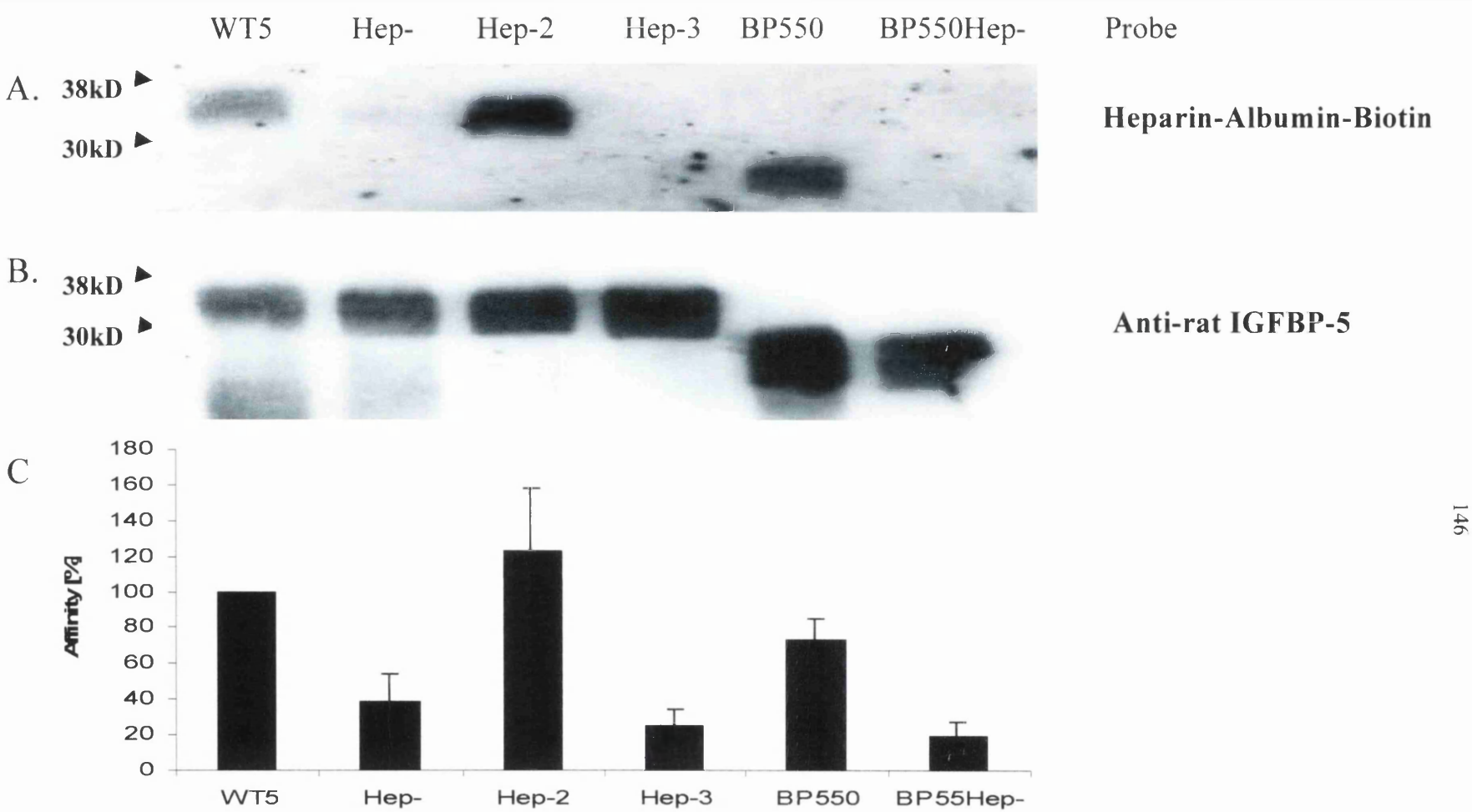


Fig V.4 Heparin ligand blot of wtIGFBP-5, Hep-, Hep-2, Hep-3, BP550 and BP550Hep-. Proteins were loaded on 15% polyacrylamide gels and run under non-reducing conditions. A. shows the results of Heparin ligand analysis, ligand reactivity was tested with heparin-albumin-biotin and streptavidin-peroxidase. Below in B. are duplicate blots used to monitor protein loading using the anti-rat IGFBP-5. C. Densitometric analysis of ligand-binding data. Levels of binding are expressed as mean of band intensity ± Standard deviation (n=3); (ligand blot/western blot) X 100%. For binding Heparin, WT5:100, Hep-: 38.7±15.8, Hep-2: 123.3±35.2, Hep-3: 25.3±9.1, BP550: 73.7±11.4, BP550Hep-: 19±9.

V.3 Discussion

Our heparin ligand blots clearly demonstrate a major loss of heparin binding for the Hep- mutant, which has four of the important C-terminal basic amino acids required for heparin binding mutated (Song *et al.* 2000). It is very interesting, therefore, that the removal of this entire basic C-terminal region, as is the case with the chimeric molecule BP552 or the truncated mutant BP550, should result in proteins with heparin binding affinities comparable to WTIGFBP-5. The heparin binding activity displayed by BP552 and BP550 must be contained within the central domain of IGFBP-5, as BP522 was unable to bind heparin. Furthermore, the work presented here suggests that heparin is prevented from interacting with these sites by the presence of the C-terminal domain of IGFBP-5.

The fact that our chimeric IGFBPs demonstrate similar affinities for binding IGFs (Chapter IV) would suggest that the N-terminal domain of IGFBP-5 is juxtaposed with the C-terminal domain of IGFBP-2 in these molecules in such a way as to simulate the spatial relationship between the N- and C-terminal domains of native IGFBP-5. However, the C-terminal domain of IGFBP-2 is unable to inhibit heparin binding to the central domain of IGFBP-5 in the BP552 chimera. One obvious difference between the C-terminal domains of IGFBP-5 and -2 is the heparin binding site between 201-218 in IGFBP-5. This initially raises the interesting possibility that heparin binding to the C-terminal site in IGFBP-5 may itself inhibit subsequent heparin binding to the central site. In support of this, others have shown that heparin binding to the C-terminal domain of IGFBP-5 does in fact reduce the activity of an IGFBP-5 specific serine protease, which cleaves within the central GAG-binding consensus sites (Imai *et al.* 1997, Nam *et al.* 1994). However, we have shown with our Hep- mutant that prevention of heparin binding to the 201-218 region of IGFBP-5 does not facilitate heparin binding to the central domain.

In order to establish the important central domain sequences required for heparin binding, we set out to simultaneously disrupt both consensus GAG-binding sites (VKKDRRKKL at residue number 132-140) proposed by Hodgkinson *et al*, 1994, by mutating both arginines in the overlapping sequence to alanines (R136A:R137A). These mutations were carried out in WTIGFBP-5, Hep- and BP550 to create the mutants Hep-2, Hep-3 and BP550Hep- respectively (Figure V.1).

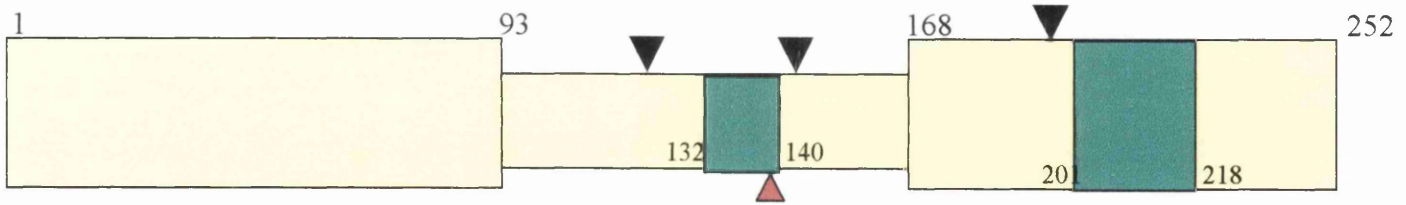
As expected, the ability of Hep-2 to bind heparin is comparable to WTIGFBP-5, since the heparin binding site in the C-terminal domain is still intactive. Conversely, Hep-3 binding to heparin resembles that of Hep- as the C-terminal site is mutated in both proteins. Overall, these results confirm that mutagenesis of the central domain heparin binding site has no appreciable effect in the presence of the C-terminal domain, which contains the major heparin binding site in the full-length protein. This would also explain why the previously reported K134A/R136A mutant of IGFBP-5 performed as well as the native protein with respect to heparin binding (Arai *et al.* 1996a), since the effect of these mutations would again be masked by the C-terminus.

The failure of BP550Hep- to bind heparin confirms that R136 and R137 are essential for heparin binding in the absence of the C-terminal domain, and pinpoints this activity to the central consensus GAG-binding region. It seems likely that heparin binding to this region of IGFBP-5 will involve several, if not all of the six basic amino acids between 132-140, although the R136A:R137A mutations do not rule out the possibility that only one of the consensus sites is active. Future work will establish the individual contribution of the basic amino acids in this region to heparin binding, in a similar fashion to the detailed mutagenesis studies performed by others on the ten basic residues between 201-218 in the C-terminus (Arai *et al.* 1996a; Parker *et al.* 1998). Also, by analogy with the 201-218 region of IGFBP-5, which binds both heparin and the ALS (Twigg *et al.* 1998), it is conceivable that the central heparin-binding site identified in this paper will also account for the central ALS binding activity of IGFBP-5 described by Twigg *et al*, 2000.

The existence of a central heparin binding site, which only becomes functionally active after the removal of the C-terminal domain, is of major significance when considering the possible functions of IGFBP-5 *in vivo*. It is particularly relevant to the proteolytic cleavage of IGFBP-5, which has been the subject of many studies. Human fibroblasts and porcine smooth muscle cells have been shown to secrete a calcium-dependent serine protease that is specific for cleaving IGFBP-5 (Imai *et al.* 1997, Nam *et al.* 1994). Peptide sequencing of the proteolytic fragments suggested K138-K139 as the primary cleavage site (Figure V.5) and this was confirmed by the creation of a protease resistant mutant of IGFBP-5 by mutating these two residues (Imai *et al.* 1997). Cleavage at this site could potentially produce an N-terminal fragment, which retains GAG-binding sites in the central domain, including R136 and R137. In addition, physiological

Fig V.5 IGFBP-5 Proteolysis

IGFBP-5
Protein (252 a.a)



= Heparin binding sites

▲ IGFBP-5 specific calcium-dependent serine protease has primary cleavage site at K138-K139.

Imai *et al* (1997) *J Clin Invest* 100: 2596-605
Nam *et al* (1994) *Endocrinology* 135: 1385-91

▼ Thrombin cleavage sites in IGFBP-5 **Zheng *et al* (1998) *Endocrinology* 139: 1708-14**

Metalloproteinases degrade both IGFBP-5 and -3. Sites of cleavage unknown.

Thrailkill *et al* (1995) *Endocrinology* 136: 3527-33

concentrations of thrombin have been shown to cleave IGFBP-5 at three sites (Figure V.5), two of which are C-terminal to the central GAG-binding site (Zheng *et al.* 1998b), and metalloproteinases have been shown to degrade both IGFBP-5 and -3 (Thraikill *et al.* 1995a). This raises the interesting possibility that the central heparin binding site in IGFBP-5 is activated *in vivo* after removal of the C-terminal domain by proteolytic cleavage. This could produce a truncated IGFBP-5 molecule that is able to bind GAGs in the ECM or on the cell surface, and which may have differential functions to the full-length protein. In support of this, it has been shown previously by others that a 23 kDa fragment of IGFBP-5, which has an identical N-terminus to the native protein, is able to stimulate osteoblast mitogenesis in the absence of IGFs (Andress & Birnbaum 1992). The ability of this fragment to bind heparin was not ascertained, but it could account for the 23 kDa fragment observed by Hodgkinson *et al*, (1994), when they eluted IGFBP complexes from a heparin affinity column.

In conclusion, we have demonstrated for the first time that there is a functional heparin binding site in the central domain of IGFBP-5 (Song *et al*, 2001). This site involves the amino acids R136 and R137, which lie within the consensus GAG-binding sites, and is active only in the absence of the C-terminal domain. Further understanding in this area will require the careful structural analysis of heparin-IGFBP-5 complexes combined with biological studies aimed at elucidating the function of IGFBP-5 proteolytic fragments.

Chapter VI. General discussion and Future study

The work undertaken in this study has provided many details relating to the molecular based interactions of IGFBP-5 with IGFs and/or ECM components (heparin). In this chapter, it is not our intention to repeat the discussion of the experimental results obtained in the thesis. Rather it is intended to discuss the possibility of using our mutant proteins to explore the role of IGFBP-5 in a biological context, particularly related to the pro-apoptotic model and IGF-independent biological actions of IGFBP-5 that were described in chapter I.

IGFBP-5 mutants

From this study, we have generated several extremely important mutants of IGFBP-5, which could now be used to test the function of this binding protein. This will first require the identification of a suitable cell culture assay, whereby the addition of IGFBP-5, either by transfection or adding recombinant protein to the culture medium, results in a measurable biological effect. When an appropriate assay is identified, it would then be possible to ask the question of whether IGF and/or ECM binding was essential for any IGFBP-5 effects by including our Double and Hep- mutants, which are non-IGF and non-ECM binders respectively. This is particularly relevant for the pro-apoptotic model for IGFBP-5 action discussed earlier, which is based upon the binding protein making contact with both ligand and ECM components simultaneously. Furthermore, proteolytic cleavage of IGFBP-5 into fragments with reduced affinity for IGFs is believed to be an additional mechanism to stimulate the activity of IGFs in biological fluids. Using the BP550 mutant, it should be possible to address the question of whether a truncated IGFBP-5, which may be representative of certain proteolytic fragments *in vivo*, has differential effects to the full-length protein. By inference, any such effects would have to be IGF-independent, and including BP550Hep- would also address the importance of ECM binding.

It is very difficult to imagine how any effects of our three chimeric mutants, BP552, BP522 and BP255, could easily be interpreted in a biological assay at this stage. This is because the loss of any IGFBP-5 sequences and /or functions in the chimeras may potentially be compensated for, or completely confused by, any gain of IGFBP-2 function. For example, the RGD sequence in the C-terminal domain of IGFBP-2 may enable BP552 and BP522 to bind cell surface integrin

receptors, thereby activating cellular responses that are completely different to those normally ascribed to IGFBP-5. Further studies of the ligand binding characteristics of these mutants may subsequently allow meaningful biological assessments of their activities.

There are several reports of *in vitro* biological systems used to identify the role of IGFBP-5, including assays in osteoblasts (Gabbitas and Canalis, 1998; Kanatani *et al*, 2000), chondrocytes (Sunic *et al*, 1998; Matsumoto *et al*, 2000), and fibroblasts (Parker *et al*, 1996; Nam *et al*, 1997; Zheng *et al*, 1998). As a start, we decided to test some of our IGFBP-5 mutants in culture systems that were being used by collaborators both within our institute and in other research organisations, and these are described below. Some progress was made towards these experiments within the period of this PhD project, although our biological experiments remain part of an ongoing study. These experiments are now discussed below.

Differentiation of C2 myoblasts

It has been shown previously by others that IGFBP-5 is the only IGFBP expressed by C2 myoblasts (James *et al*., 1993), which makes these cells an attractive model for studying IGFBP-5 function without potential interference from other IGFBPs. Furthermore, it was shown that the binding protein was secreted within 12h of the onset of terminal differentiation, suggesting a role for IGFBP-5 in this process (James *et al*, 1993). In support of this, it was subsequently shown that over expressing IGFBP-5 in these cells stimulates cell proliferation, thereby inhibiting cell differentiation, and it was shown that this effect was dependent upon the presence of IGFs (James *et al*, 1996). These authors postulated that IGFBP-5 in the ECM may function as a source of sustained low level growth factor release (James *et al*, 1996). This myoblast differentiation assay is being used successfully in the laboratory of Dr Claire Stewart at the University of Bristol, who has supplied us with untransfected C2 cells. We have clonally selected G418 resistant lines transfected with WTIGFBP-5, Double and Hep- constructs and sent these lines for analysis in Bristol. We would predict that the over expression of the Double mutant in these cells would have no effect on cell differentiation as the mutant is unable to bind IGFs. However, it is of interest to us to examine how the Hep- mutant will behave, as this will indicate whether IGFBP-5 must bind to the ECM in order to carry out its effects on differentiation. Our lines have indeed been shown to over express

the wild-type and mutant IGFBP-5 proteins, and preliminary data suggests that the Hep- mutant performs as well as WTIGFBP-5 in this assay, indicating that ECM binding may not be important (pers comm M. Grohmann).

Mammosphere tissue culture system

Work from other laboratories has shown that the culture of primary mammary epithelial cells on Engelbreth-Holm-Swarm (EHS) matrix, results in the formation of three-dimensional multi-cellular structures termed mammospheres, which contain a central luminal space into which milk proteins are secreted (Li *et al*, 1987; Barcellos-Hoff *et al*, 1989; Wilde *et al*, 1995; Roskelley *et al*, 1994). Formation of the luminal space in the mammospheres has been shown to involve programmed cell death of mammary epithelial cells, and consequently, it has been proposed that the mammosphere may provide a useful model for the apoptosis of mammary epithelial cells that occurs during involution of the mammmary gland (Blatchford *et al*, 1995 and 1999; Hurley *et al*, 1994; Barcellos-Hoff *et al*, 1989; Streuli *et al*, 1991). Interestingly, from our point of view, it has also been observed that there is high levels of IGFBP-5 expression in the culture media during mammosphere formation (Blatchford *et al*, 1999), and preliminary immunocytochemistry experiments have shown the presence of IGFBP-5 protein in the luminal space, (pers. comm. D Flint), suggestive of a role for the binding protein in development of this structure. Therefore, we initially considered mammosphere culture as a potentially useful *in vitro* system in which to test the pro-apoptotic model for IGFBP-5 action.

A serious problem with this strategy is the reported very low transfection efficiencies (less than 0.3%) of both mouse and goat primary mammary epithelial using standard lipofectin based techniques (Wells., 2000). Therefore, as a prerequisite to expressing WTIGFBP-5 and various mutants in these cells prior to mammosphere differentiation, we had to devise an alternative strategy of delivering promoter-gene constructs into this system. We decided to use the baculovirus protein expression system, which is being used increasingly to express proteins in mammalian cells. Although the receptor for baculovirus on mammalian cells is not known, it is likely to be a glycosaminoglycan or other ubiquitous cellular component, given the wide range of cell types susceptible to infection. We have constructed a hybrid mammalian-baculovirus expression vector (Figure VI.1; described in detail in chapter II), and have used this to generate a reporter virus,

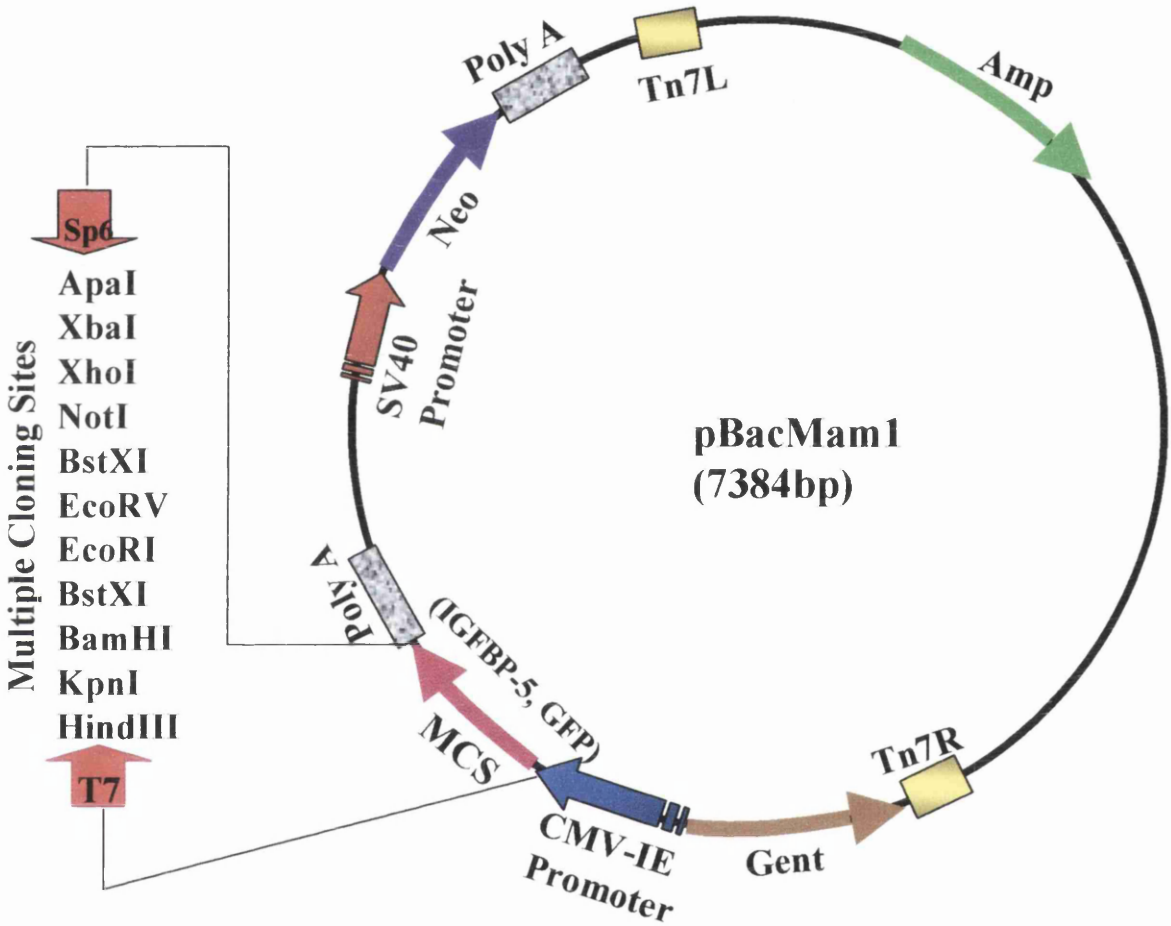


Fig VI.1 pBacMam1. A baculovirus mammalian expression vector, which contains a multiple cloning site (MCS) down stream of the CMV-IE promoter. This cassette also contains the gene for G418 (Neo) and gentamycin (Gent) resistence and is flanked by transposable elements (Tn7L and Tn7R) which allow transposition into Bacmid DNA (see chapter II).

which expresses green fluorescence protein (GFP) from the cytomegalovirus (CMV) immediate early promoter (a powerful promoter in mammalian cells). The results of infection of both primary goat mammary epithelial cells and C2 myoblasts in culture with this virus at different multiplicities of infection and time post infection are shown in Figure VI.2 and Figure VI.3 respectively, with GFP expression evident in both lines. From this we estimate an infection efficiency of the primary goat mammary epithelial cells as approximately 20%, which is much higher than that produced from traditional transfection techniques.

Based on this, we went ahead and produced BacMam virus with the potential to express WTIGFBP-5, Double and Hep- from the CMV promoter. However, the effects of infection of primary goat mammary epithelial cells with these viruses is the subject of future work. If endogenous IGFBP-5 is involved in the apoptosis of mammary epithelial cells that takes place during mammosphere lumen formation, we can hypothesise the possible effects of over- expression of WTIGFBP-5, Double and Hep- in this system, based on the pro-apoptotic model, discussed before. Briefly, expression of exogenous WTIGFBP-5 may simply amplify the effects of the endogenous protein (Figure VI.4.A), although it is conceivable that this could affect the timing of lumen formation. However, we postulate that the expression of either Double or Hep- could possibly compete with the action of endogenous IGFBP-5 in this system, and consequently delay or inhibit lumen formation. Here the Double mutant protein could fill up available sites in the ECM, while unbound to IGFs, (Figure VI.4.B), while the Hep- mutant could still bind IGFs, but could not then sequester them to the ECM (Figure VI.4.C). Either way, IGFs may then become available for interaction with the type 1 receptor following proteolysis of the unprotected binding protein, and this, in turn, would interfere with the onset of apoptosis. These hypotheses require experimental validation, but are clearly testable.

Transgenics

Depending upon the effects of our IGFBP-5 mutants in appropriate *in vitro* systems, we would propose to test our IGFBP-5 mutants *in vivo* using transgenic mice. In collaboration with Dr Bruce Whitelaw at the Roslin Institute, our laboratory has already generated transgenic mice expressing wild type rat IGFBP-5 from the beta lactoglobulin (BLG) promoter, so that the transgene is specifically expressed in the mammary gland. It has been verified that transgenic mice

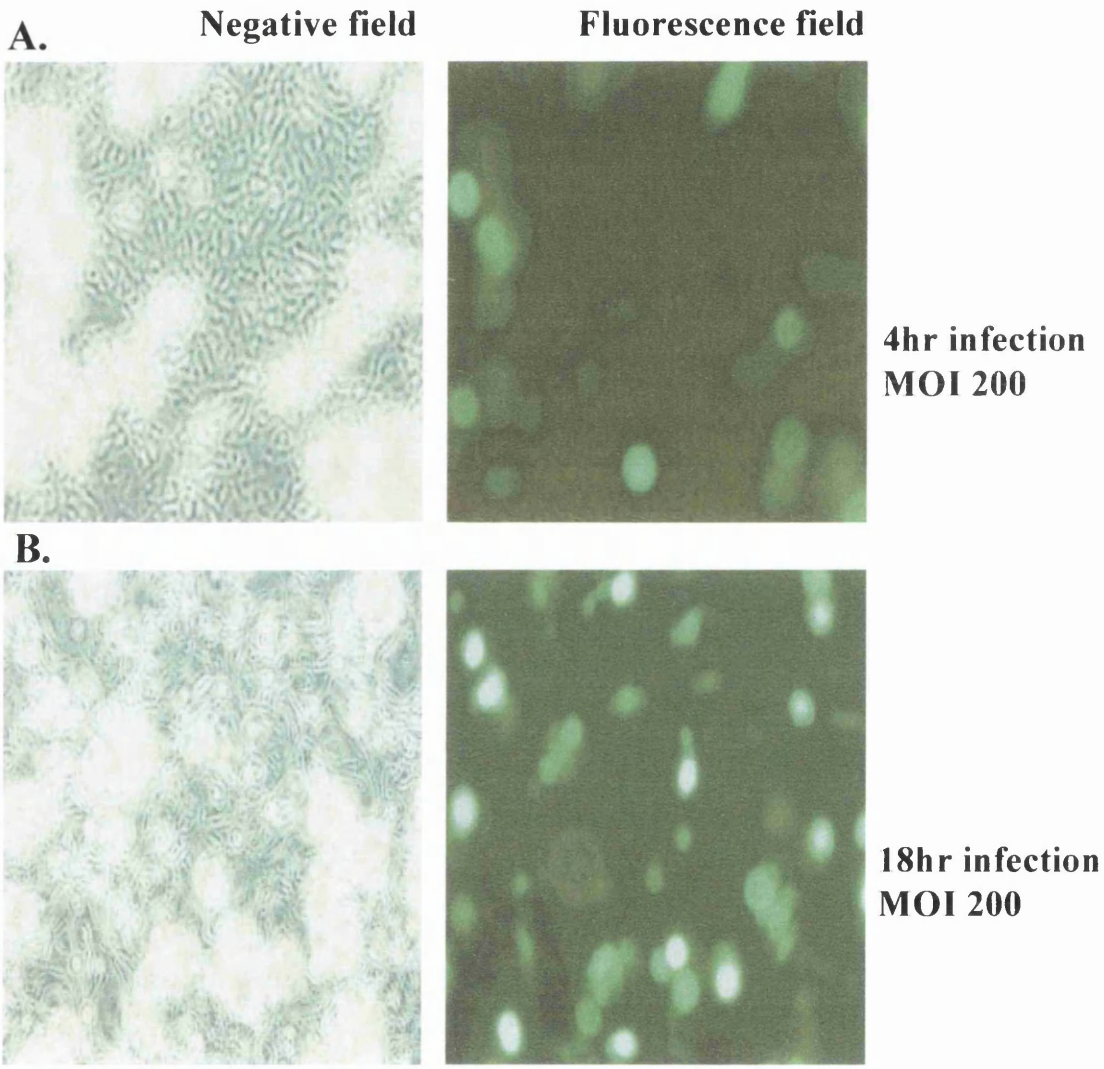


Fig VI.2 Transfection of primary goat mammary epithelial cells with pBacMam1 GFP reporter baculovirus. Cells were observed under normal light (Negative) and fluorescence. (A.) shows a multiplicity of infection (MOI) of 200, 4hr post infection. (B.) shows a MOI of 200, 18hr post infection.

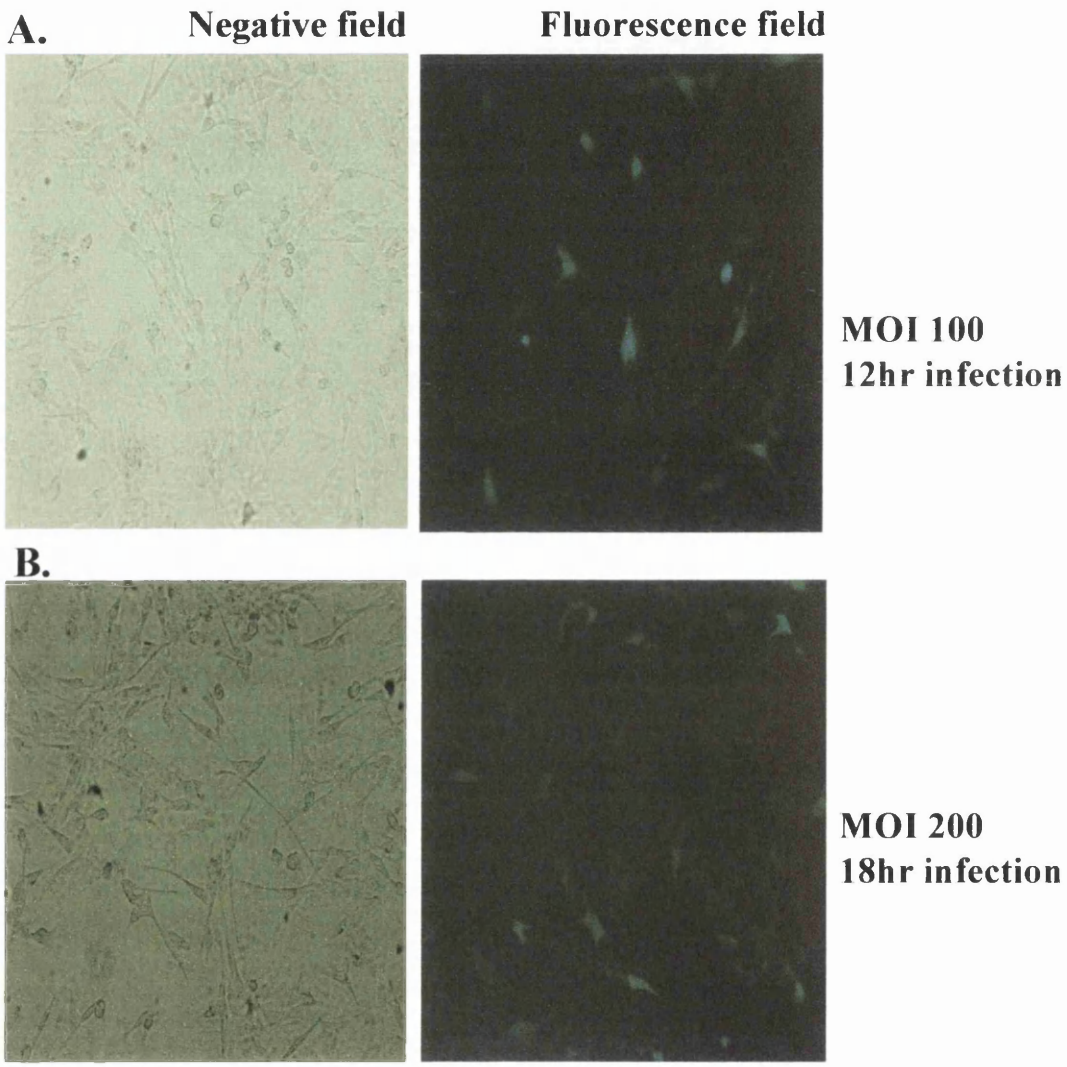


Fig VI.3 Transfection of C2 myoblasts with pBacMam1 GFP reporter baculovirus. Cells were observed under normal light (Negative) and fluorescence. (A.) shows a multiplicity of infection (MOI) of 100, 12hr post infection. (B.) shows a MOI of 200, 18hr post infection.

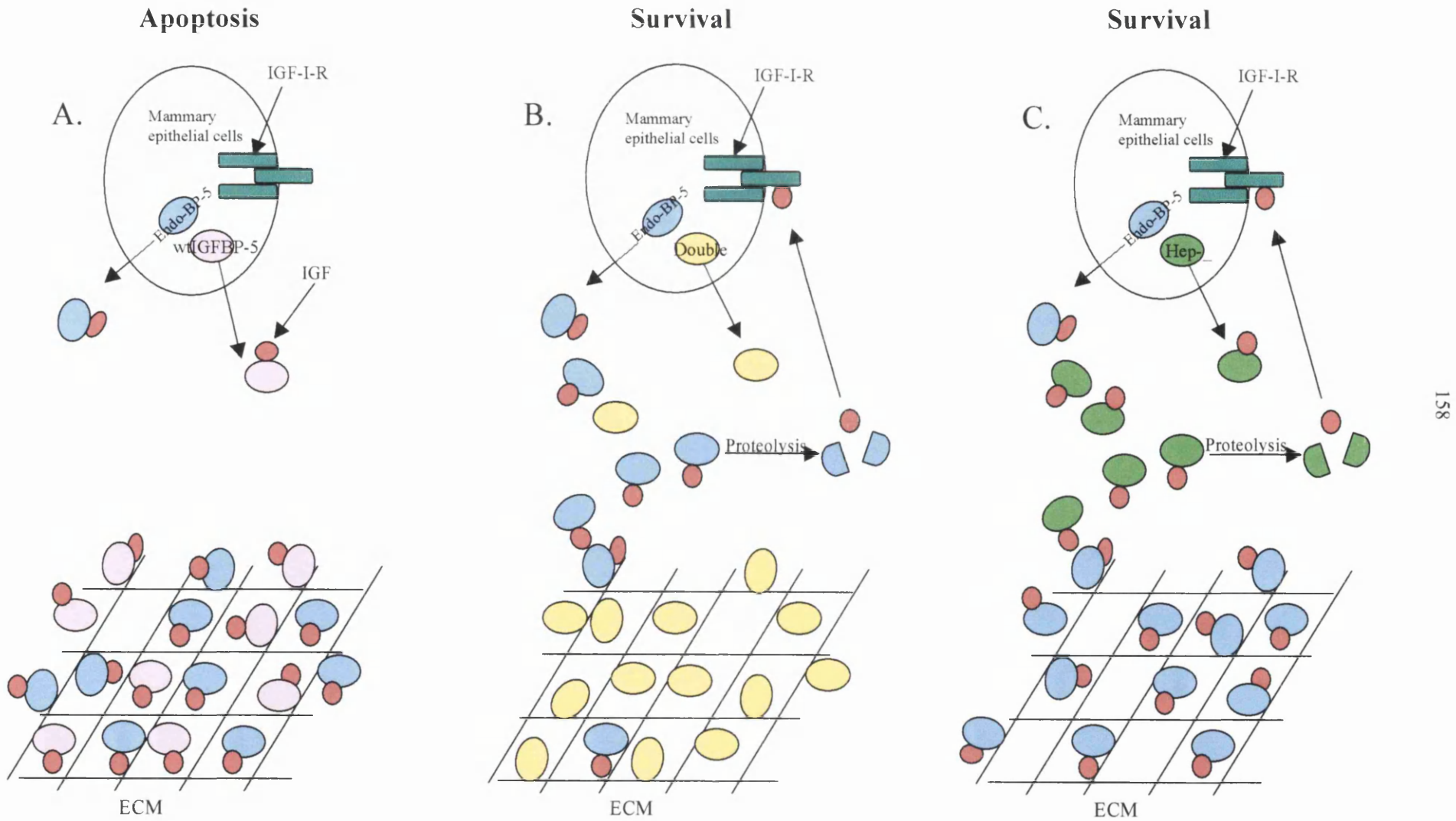


Figure VI.4 Schematic outlining a model of the effect of exogenous (A) wtIGFBP-5, (B) Double mutant and (C) Hep- mutant expression on the function of endogenous IGFBP-5 (Endo-BP-5)

have high levels of IGFBP-5 expression in the mammary gland during lactation, whereas the binding protein is only expressed at the onset of involution in the wild type controls (pers. comm. D. Flint). Based on the pro-apoptotic model it has been postulated that this aberrant expression of IGFBP-5 may result in premature involution of the gland. In support of this, we have found that the transgenic mice have impaired mammary gland development and produce less milk. Applying our model for a competitive effect of either the Double or Hep- mutant on endogenous IGFBP-5 action (Figure VI.4), it is conceivable that expression of these mutants in transgenics in an analogous fashion to that described above for WTIGFBP-5, could have the opposite effect and result in extended lactation of the mammary gland.

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