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The Function and Regulation of Insulin-like Growth Factor Binding Protein-5 in HC11 cells

Alice Miwook Sorrell

Biochemistry and Molecular Biology
Institute of Biomedical and Life Science

Thesis submitted in accordance with the requirements for the degree of Doctor of Philosophy

Hannah Research Institute, Ayr
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Declaration

The work contained in this thesis was carried out by myself, unless indicated otherwise in the text, at the Hannah Research Institute under the supervision of Dr. Gordon. J. Allan and Dr. David. J. Flint. No part of this work has been submitted for consideration for any other degree or award.

Alice Miwook Sorrell (formerly known as Miwook A. Park)

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Finally, I wish to express my love and gratitude to all my family. I am forever grateful to my parents for their unconditional support and affection at each turn of the road. I would like to thank my parents-in-law for their love and support. This thesis is dedicated to David for being my husband and for putting up with me while I wrote this thesis; thank you for your understanding, patience, support and love.

Abstract

Our group has previously shown that IGFBP-5 expression increases dramatically during involution of the mammary gland and subsequently we developed a transgenic mouse model expressing IGFBP-5 specifically in the mammary gland, which proved that the binding protein was a causative factor in mammary epithelial cell death. In this study, the aim was to develop an *in vitro* system to investigate the regulation of IGFBP-5 expression and the function of the binding protein on cell death/survival and tissue remodelling. The mouse mammary epithelial cell line, HC11, was chosen for this and initially we characterised the IGFBP expression profiles in these cells. We demonstrated that although IGFBP-5 protein levels are up-regulated by up to 10-fold during differentiation of HC11 cells induced by treatment with a lactogenic hormone mix (DIP) and that IGFBP-2 secretion was down regulated, there was a clear dissociation between the process of cell differentiation and the regulation of these IGFBPs. Furthermore, the mRNA expression profile of the IGFBPs was also established using quantitative RT-PCR to examine similarities and differences in IGFBP mRNA expression profiles between the mammary gland and the HC11 cell line. In addition to the significant up-regulation of IGFBP-5 message in differentiated HC11 cells, we report that IGFBP-5 mRNA levels were increased by a dramatic 54-fold in the involuting mouse mammary gland. We decided to study the DIP-induced increase in IGFBP-5 levels in HC11 cells as an *in vitro* model in which to study the potential molecular signals responsible for the induction of IGFBP-5 expression in the involuting mammary gland. Using transient gene transfer methods, we demonstrated that there is an enhancer element(s) between positions −1004 to −156 in the IGFBP-5 promoter that results in significant induction of gene expression in HC11 cells and that there is a potential site for a novel transcriptional regulator(s) of IGFBP-5 expression at position -556, which merits further investigation. As HC11 cells also secrete a plasminogen activator *in vitro*, which results in cleavage of focal adhesions and cell death, and because IGFBP-5 has been previously shown to bind to PAI-1, we decided to use this system to determine the biological consequences of IGFBP-5/PAI-1 interaction. Our data demonstrates that IGFBP-5 can induce cell death both by sequestering IGF-I and by activation of plasmin which, in turn, induces degradation of the extracellular matrix. We have also shown that IGFBP-5 can enhance the

activation of tPA, in a PAI-1- and IGF-independent fashion and that this can result in cell death. These studies thus identified the possibility that IGFBP-5 may act as a central coordinator of the apoptosis and ECM degradation that occurs during tissue remodelling.

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List of abbreviations

ALS	Acid labile subunit
AMV	avian myeloblastosis virus
AP	activation protein
BAD	Bcl-xL/Bcl-2-Associated Death Promoter
Bcl-2	B-cell lymphoma 2
Bcl-xL	long Bcl-x isoform
BLG	β-lactoglobulin
BMP-7	bone morphogenetic protein-7
bp	basepairs
BSA	bovine serum albumin
cAMP	cyclic activated protein kinase
C/EBP	CCAAT enhancer binding protein
CHO	Chinese hamster ovary
cpm	Counts per minute
CrK	Ca -dependent protein-kinase (CDPK)-related protein kinase
DIP	dexamethasone (D), insulin (I), and prolactin (P)
DMEM	Dulbecco's modified Eagle's medium
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
EV	pGEX 6P-1 empty vector
FAK	focal adhesion kinase
FCS	fetal calf serum
FGF	fibroblast growth factor
GAG	Glycosaminoglycans
GH	Growth Hormone
Grb-2	growth factor receptor-bound protein 2
GST	glutathione *S*-transferase
HBSS	Hanks Balanced Salt Solution
HOX	Homeobox gene
HRP	Horse radish peroxidase
IL-3	Interleukin 3
IGFBP	Insulin-like growth factor binding protein
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor-II

IGF-IR	type I IGF receptor
IGF-IIR	type II IGF receptor
IPTG	isopropyl-β-D-galacosidase
IRF-1	interferon regulatory facor-1
IRS-1	Insulin receptor substrate-1
kDa	kilo Dalton
LB	Luria-Bertaini
MAP	Ras-Raf-Mitogen Activated Protein
MMPs	matrix metalloprotcinascs
mt	mutant
mRNA	messenger RNA
Myb	Myeloblast
MYC	myelocytomatosis oncogene
NF-1	nuclear factor-1
NLS	nuclear localisation signal
NMR	nuclear magnetic resonance
PA	plasminogen activator
PAI-1	plaminogen activator inhibitor-1
PBS	Phosphate-buffered saline
PDGF	platelet derived growth factor
PFA	Paraformaldehyde
PGE2	prostaglandin E2
Pgen	plasminogen
PI-3 K	phosphatidylinositol-3 kinase
PKB	protein kinase B
PTEN	phosphatc and tensin homologue
PTH	parathyroid hormone
RGD	Arg-Gly-Asp
RIA	Radioimmunoassay
RP-HPLC	Reverse Phase- High Performance Liquid Chromatography
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
SF	serum-free
SH3 domain	shr homology 3
Shc	Src homology collagen
SOS	son of sevenless protein
SP1F	GC box factor SP1/GC
STAT	transducers and activators of transcription

TFA	trifluoroacetic acid
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α
tPA	type plasminogen activator
uPA	urokinase plasminogen activator
VLL-pNA	H-Val-Leu-Lys-p-nitroanilide
VSMC	vascular smooth muscle cells
wt	Wild type
ZBP-89	zinc finger transcriptional factor

Publications

(Note: Alice M. Sorrell was formerly known as M. A. Park)

Phillips, K., Park, M. A., Quarrie, L. H., Boutinaud, M., Lochrie, J. D., Flint, D. J., Allan, G. J. and Beattie, J. (2003). Hormonal control of IGF-binding protein (IGFBP)-5 and IGFBP-2 secretion during differentiation of the IIC11 mouse mammary epithelial cell line. *J Mol Endocrinol* **31**, 197-208.

Boutinaud, M., Shand, J. H., Park, M. A., Phillips, K., Beattie, J., Flint, D. J. and Allan, G. J. (2004). A quantitative RT-PCR study of the mRNA expression profile of the IGF axis during mammary gland development. *J Mol Endocrinol* **33**, 195-207.

Alice M Sorrell, John H Shand, Elizabeth Tonner, Matteo Gamberoni, Pier A Accorse, James Beattie, Gordon J Allan and David J Flint (2005). IGFBP-5 activates plasminogen by interaction with tissue plasminogen activator, independently of its ability to bind to plasminogen activator inhibitor-1, IGF-I or heparin. *Submitted in J Biol Chem.*

Chapter I. Introduction

I.1 Structure and Function of IGFBP-5

I.1.1 IGF-axis

Insulin-like growth factors (IGF-I and IGF-II), single chain polypeptides that share significant structural homology with insulin, are mitogenic and potent survival factors present in the circulation (reviewed by Baserga et al., 1997). They are secreted by many cell types and have multiple biological functions in proliferation, differentiation and other anabolic responses, including resistance to apoptosis (Baserga et al., 1997; Stewart et al., 1996).

The biological effects of IGFs are mediated through binding to high-affinity receptors on the cell surface. The type I IGF receptor (IGF-IR), which is structurally closely related to the insulin receptor, is a disulphide linked heterotetrameric protein complex containing a tyrosine kinase domain that mediates signal transduction pathways (De Meyts et al., 1994). IGF-IR is believed to mediate IGF action by the phosphorylation of cellular substrates, which specialise in growth and differentiative functions (Cheatham, 1995; LeRoith et al., 1995). The structurally distinct type II IGF receptor (IGF-IIR) lacks tyrosine kinase activity and is actually identical to the cation-independent mannose 6-phosphate receptor (Kornfeld, 1992). 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 IGF-IR (Czech, 1989). However, although IGF-II binds IGF-IIR with greater affinity than IGF-I, IGF-IIR appears to be involved in the mediation of IGF-II degradation and targeting of lysosomal enzymes to lysosomes (Wang et al., 1994). Inactivation of IGF-IIR in mice by gene targeting results in foetal overgrowth, skeletal abnormalities, and perinatal death due to overexposure of the foetus to IGF-II (Lau et al., 1994; Ludwig et al., 1996).

The observation that most of the IGFs present in serum migrate in higher molecular mass fractions, while the molecular mass of free IGFs is approximately 7.5 kDa, led investigators to

propose the existence of carrier proteins. These carrier proteins were postulated to form a complex, which maintains a circulating reservoir of IGFs, transporting and prolonging the half-life of the growth factors. These carrier proteins, which were revealed to be IGFBPs, regulate IGF distribution to the tissues by preventing them from binding to the receptors (Zapf et al., 1979). All six IGFBPs are found in the circulation either in the free form or in binary complexes with IGFs. Free or binary-complexes of IGFBPs are believed to exit the circulation rapidly, whereas ternary complexes with the Acid-labile subunit (ALS) appear to be essentially confined to the vascular compartment (Guler et al., 1989; Lewitt et al., 1994; Young and Clemmons, 1994).

The most abundant circulating IGFBP for transporting IGFs is IGFBP-3. It carries 75 % or more of serum IGF-I and -II in heterotrimeric complexes that also contain the ALS, which is a protein of approximately 85 kDa molecular weight (Baxter, 1988). IGFBP-5, which is present at about 10 % of the molar concentration of IGFBP-3, can also form ternary complexes with the ALS (Twigg and Baxter, 1998). Approximately 90 % of IGFBP-3 and 55 % of IGFBP-5 present in the circulation are in these binary or tertiary complexes in healthy adults (Baxter et al., 2002).

IGFBPs exert a complex array of functions at the cellular level (Baxter et al., 2002). There is little information on the exact relationship between IGFBPs in the circulation and those in the cellular environment, but it appears that the IGFBPs may be differentially targeted to different tissues depending on both their primary structure and their post-translational modifications (Baxter et al., 2002). IGFBPs in the circulation can be expected to have both important autocrine and paracrine effects. As well as modulating activation of the IGF-IR by IGFs (Jones et al., 1993a; Karas et al., 1997; Ricort and Binoux, 2001), IGFBPs are documented to also affect cell motility and adhesion (Jones et al., 1993a; Perks et al., 1999b), apoptosis and survival, and cell cycle regulation in an IGF-independent manner (Firth et al., 1998a; Miyake et al., 2000; Rajah et al., 1997).

A schematic diagram shown in Figure I.1 illustrates the major components of the IGF axis. The IGF axis consists of small peptide hormones, IGF-I, IGF-II, their receptors (the type I and type II IGF receptors) and six known IGF binding proteins (IGFBP-1 through -6). The IGFs bind both

their receptors and binding proteins with high affinity. The IGFBPs are able to modulate the action of IGFs in several ways, including an inhibitory model in which IGFBPs sequester IGFs from their receptors, an enhancing model in which IGFBPs transport IGFs to their site of action, or by a receptor-independent model that may involve direct interaction of IGFBPs with IGFBP receptors (Clemmons, 1997). The modulation of IGF levels by IGFBPs is further regulated by IGFBP proteases which cleave the high affinity IGFBPs into fragments with lower affinity for IGFs, thereby increasing free IGF bioavailability. This process leads to reduced inhibition of cell growth by IGFBPs. Finally, some IGFBPs also have been shown to bind to other cellular components, and cell surface receptors have been identified for both IGFBP-1 and -3. IGFBP-5 can also bind to the extra cellular matrix with high affinity and this will be discussed in more detail later.

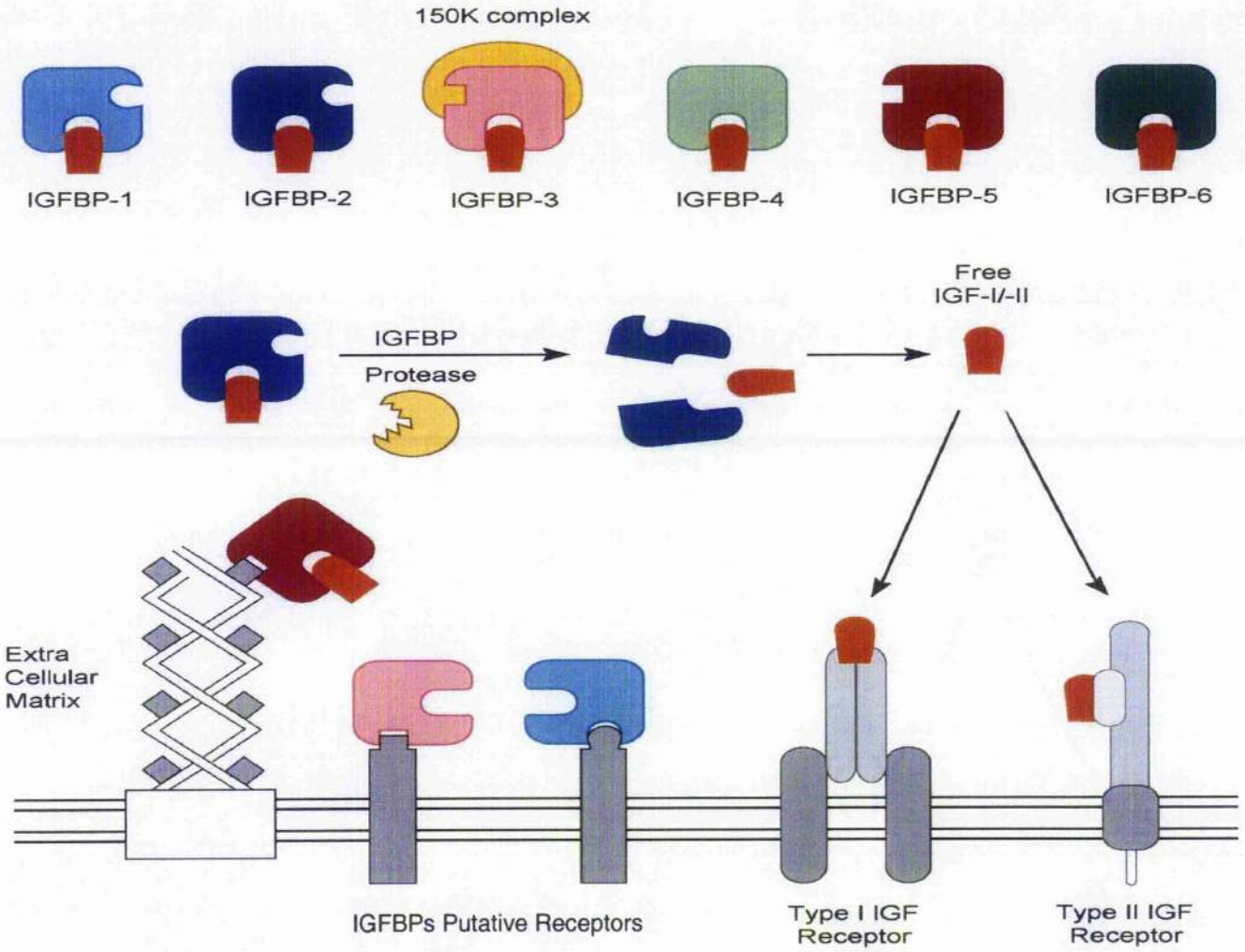


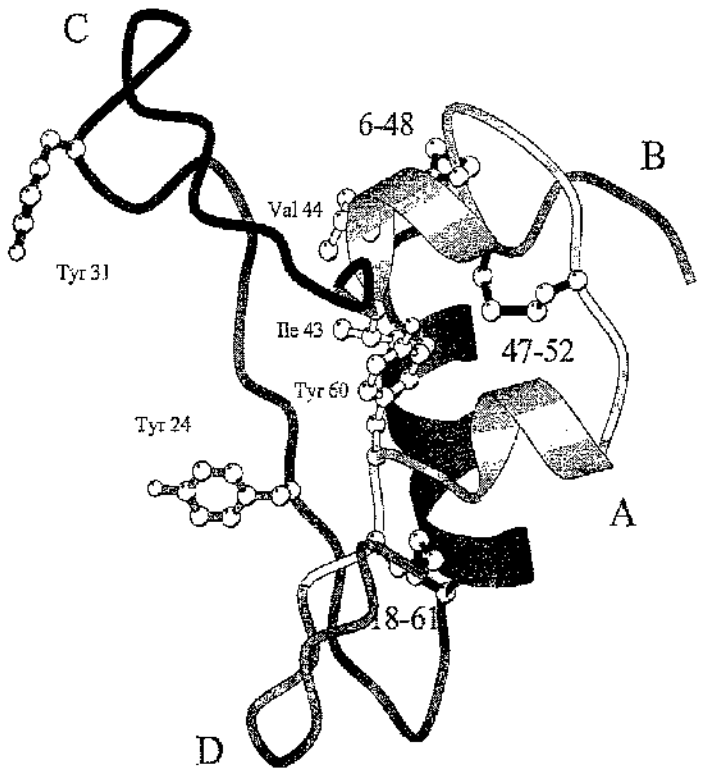
Figure I.1 The components of the IGF axis.

I.1.1.1 Insulin-like growth factor (IGFs)

I.1.1.1.1 Structure

It has been well demonstrated that the IGFs stimulate a wide variety of cells to proliferate, differentiate, and exhibit many other anabolic responses, including resistance to apoptosis (Baserga et al., 1997). IGF-I and -II, which are structurally similar to insulin, are two highly homologous small hormone peptides of approximately 7 kDa molecular mass. 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 Humbel, 1976; Rinderknecht and Humbel, 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). IGF-I has many growth-promoting and metabolic activities (Froesch and Zapf, 1985). The first 29 residues of IGF-I are homologous with the B-chain of insulin (B-region; 1–29), the following 12 residues are analogous to the C-peptide of pro-insulin (C-region; 30–41) and the next 21 residues are homologous to the A-chain of insulin (A-region; 42–62). The carboxy-terminal octapeptide (D-region; 63–70) has no counterpart in the insulin molecule. In the absence of a crystal structure, the tertiary structure of IGF-1 has been modelled on that of porcine insulin (Figure I. 2) (Blundell et al., 1983; Blundell et al., 1978). 2-D NMR studies have confirmed that the solution structure of IGF-1 is consistent with this model (Cooke et al., 1991; Sato et al., 1993). In the insulin fold an A-chain of 21 residues and a B chain of 30 residues are cross-linked by two disulphide bridges (A20–B19 and A7–B7) on either side of the B-chain α-helix. A third intrachain disulphide (A6–A11) bridges the loop between the two short anti-parallel A-chain helices (Gill et al., 1999). The corresponding disulphide connectivity (18–61,6–48,47–52) has been confirmed for plasma-derived human IGF-1 (Axelsson et al., 1992). IGFs exert their diverse biological effects through interaction with specific cell-surface receptors.

A. IGF-I



B. Insulin

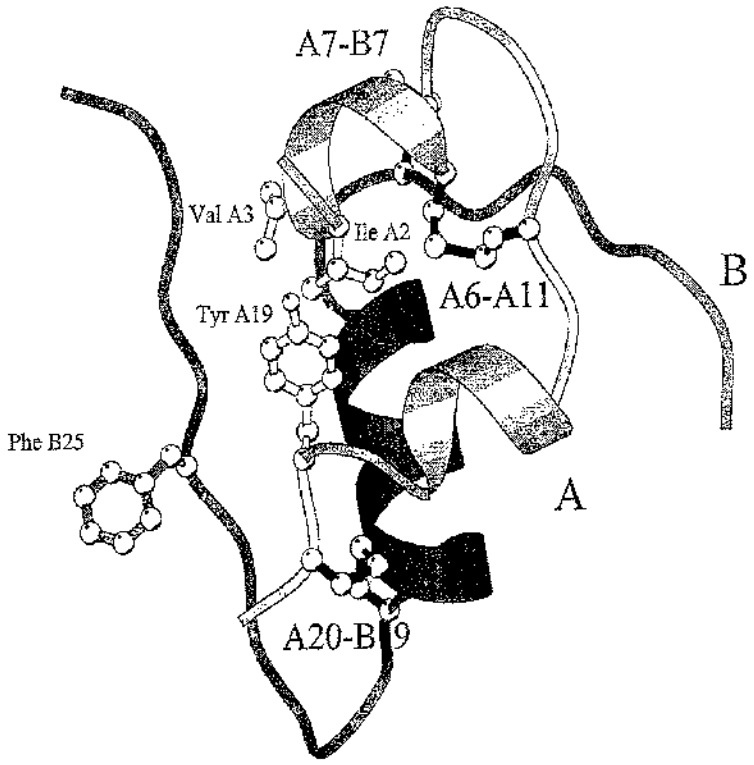


Figure I. 2 Tertiary structure of IGF-1 and Insulin. A. IGF-I The B- and D-regions are in light grey, the C-region is in dark grey and the A-region is in white. The side chains of the insulin receptor binding determinants (Tyr24 and Val44) and the IGF-1 receptor binding determinants (Tyr24 and Tyr31) are also shown. **B. Insulin** The B-chain is in light grey and the A-chain is in white. The side chains of the insulin receptor binding determinants (B25 Phe and A3 Val) are also shown (Gill et al., 1999).

I.1.1.1.2 ***In vitro* function**

Metabolic and mitogenic effects The IGFs exert acute metabolic effects on protein and carbohydrate metabolism on most cell types via the widely expressed IGF-IR. IGF-I stimulates amino acid transport, glucose utilisation, lipid formation, and protein synthesis, though its effects vary depending on cell type (Miers and Barrett, 1998). In skeletal muscle the major *in vitro* effect of IGF-I is in the stimulation of glucose uptake, glycogen synthesis and glycolysis (Dimitriadis et al., 1992).

Promotion of cell cycle progression The ability of IGFs to stimulate DNA synthesis has been widely studied. Competence factors, such as platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) induce quiescent cells to enter G_1 and IGF-1 functions as a progression factor late in G_1, allowing cells to continue into DNA synthesis and proliferation (Pardee, 1989). Since PDGF increases the numbers of IGF-IR in fibroblasts (Clemmons and Shaw, 1983) and receptor over-expression bypasses the need for PDGF, the function of PDGF as a competence factor may be to increase IGF-IR levels (Baserga and Rubin, 1993). Likewise, haemopoietic IL-3 dependent cells over-expressing IGF-IR become IL-3 independent in the presence of excess IGF-1 (McCubrey et al., 1991). As might be expected from its role in cell cycle progression, IGF-I induces mitogenesis in a wide variety of cell types (Baserga et al., 1997).

Anti-apoptotic effect As discussed above, IGFs stimulate a mitogenic response in many cell types, however in haematopoietc cells and in some carcinoma cell lines they can function as survival factors preventing apoptosis (reviewed in (LeRoith and Roberts, 1993). Proliferation and apoptosis signals may share some common pathways as they both induce similar changes in the morphology of the cells such as chromatin condensation, loss of cell-cell contact inhibition, and nuclear disintegration. This is supported by the observation that over-expression of proteins normally associated with cell proliferation can cause apoptosis (Hartwell and Kastan, 1994; Steller, 1995). However, IGF-I has been shown to support viability in non proliferating cells in culture

(Beck, 1994; Calsson-Skwirut et al., 1989; LeRoith et al., 1993) suggesting that its stimulation of cell proliferation and anti-apoptotic action may be two distinct signals (Rubin and Baserga, 1995).

I.1.1.1.3 ***In vivo* function**

Anabolic insulin-like effects The *in vivo* effects of IGFs are mainly insulin-like effects (stimulation of glucose uptake and glycogen synthesis) in fat and muscle cells (Froesch and Zapf, 1985).

Growth promoting effect through mediation of Growth Hormone (GH) effects. As a result, IGFs appeared to stimulate body weight gain and skeletal muscle elongation (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, 1988), bone endothelial cells (Fiorelli et al., 1994), chondrocytes (Ohlsson et al., 1992), hormone synthesis in ovary (Christman et al., 1991; Davoren et al., 1985; Erickson et al., 1991; Hernandez et al., 1988; Talavera and Menon, 1991), spermatogonial cells in testes (Soder, 1992), and various cancer cells (reviewed by (LeRoith et al., 1995).

I.1.2 IGF receptors and signalling

The two IGF receptors, IGF type I and type II receptors are found on most cell surfaces, but, to date, most of the biological actions of the IGFs have been attributed to interaction with IGF-IR.

I.1.2.1 IGF type I receptor

The IGF-I receptor (IGF-IR) is ubiquitously expressed, with highest levels seen during embryonic development and organogenesis (Allan et al., 2001 for review). 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 (Baker et al., 1993; Liu et al., 1993a).

The IGF-IR 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 mRNA derived from the single 21-exon IGF-IR gene, located on chromosome 15q25-q26 (Abbott et al., 1992; Ullrich et al., 1986).

The α-subunits contain the ligand-binding region of the receptor. The IGF-IR 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; LeRoith 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 (LeRoith 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, which leads to phosphorylation of other important tyrosines on the receptor as well as on endogenous substrates (reviewed by (Petley et al., 1999).

I.1.2.2 IGF type II receptor

The IGF-II receptor (IGF-IIR) 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-IIR binds ligand, and the cytoplasmic region encodes segments responsible for interaction with

different subcellular compartments (Lobel et al., 1988; Rohrer et al., 1995), including those involved in endocytosis (Lobel et al., 1988), and potentially for coupling to inhibitory GTP-binding proteins (Nishimoto et al., 1987; Okamoto et al., 1990). The IGF-IIR 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 residue repeats that share 16-38% identity (Lobel et al., 1988; MacDonald et al., 1988; Morgan et al., 1987; Szebenyi and Rotwein, 1994; Zhou et al., 1995). However, as mentioned before, most of the effects of IGF-II are thought to be mediated via IGF-I receptor (Louafi et al., 2003).

1.1.2.3 Signalling pathways

The signal pathways leading to the different cellular responses (primarily proliferation and differentiation) of cells to the IGFs are distinct (reviewed by Petley et al., 1999). In addition, differences in timing as well as in signalling pathways, might account for the variation in the cellular responses (reviewed by Petley et al., 1999). For example, it has been demonstrated that in muscle cells, in addition to the differences in signalling pathways leading to proliferation and differentiation, their responses to IGF are also separated temporally; the proliferation response occurs first, followed by differentiation (Coolican et al., 1997; Ewton et al., 1998).

The pathways involved in the mitogenic response to IGF differ dramatically from one cell type to another. Some cell types, such as myoblasts and adipocytes, clearly employ the Ras-Raf-Mitogen Activated Protein (MAP) kinase signalling pathway for cell proliferation (Coolican et al., 1997; Valverde et al., 1996), while others, such as MCF-7 mammary tumours and brain capillary cells, proliferate in response to signals mediated by phosphatidylinositol-3 (PI-3) kinase (Dufourny et al., 1997). Similarly, most of the systems studied use a PI-3 kinase pathway during IGF-mediated differentiation, although some others, such as SH-SY5Y neuroblastoma cells, differentiate in response to the MAP kinase pathway (Petley et al., 1999). Thus, it seems that there

are no simple generalisations that can be used to forecast the signalling pathway that will be involved in any response to the IGFs.

Major pathways of Insulin/IGF signalling have been well established, as illustrated in Figure I. 3. Binding of IGF-I to the extracellular α-subunit of IGF-IR results in the activation of the tyrosine kinase domain of the receptor β-subunits by phosphorylation, and increased tyrosine phosphorylation of several downstream substrate molecules, such as IRS-1, Shc and Crk (Rubin and Baserga, 1995). Each one of these proteins can then bind several other protein substrates so that the signal cascade proceeds down the pathway, but it is also propagated horizontally to other pathways. The IRS proteins have no intrinsic enzymatic activity, but are thought to act as a link between the activated receptor and downstream components of the signalling cascade.

Probably the most important pathway through which IGF-I can exert its mitogenic effects is the activation of the MAP kinase pathway (Rubin and Baserga, 1995). Activation of the MAP kinase pathway is initiated by growth factor receptor bound-2 receptor (Grb-2) binding to IRS-1, and the subsequent binding of SOS to this complex via SH3 domains. This is followed by activation of Ras and Raf and of downstream MAP kinase. The result is transcriptional activation of numerous genes involved in the regulation of cell division and proliferation.

In addition, activated IRS-I also binds the p85 subunit of PI 3 kinase (PI-3K) and stimulates the activity of the p110 catalytic subunit of PI-3K. The activated p110 then phophorylates phophoinositides, which trigger the phosphorylation of the serine kinase, protein kinase B (PKB/Akt). This in turn phosphorylates Bcl-xl/Bcl-2 associated death promoter (BAD), an apoptosis-inducing member of the B-cell lymphoma 2 (Bcl-2) family of proteins. BAD becomes sequestered by 14-3-3 proteins and is unable to bind and inactivate anti-apoptotic Bcl-2 and long form of Bcl-x isoform (Bcl-xl), thus resulting in the suppression of apoptosis.

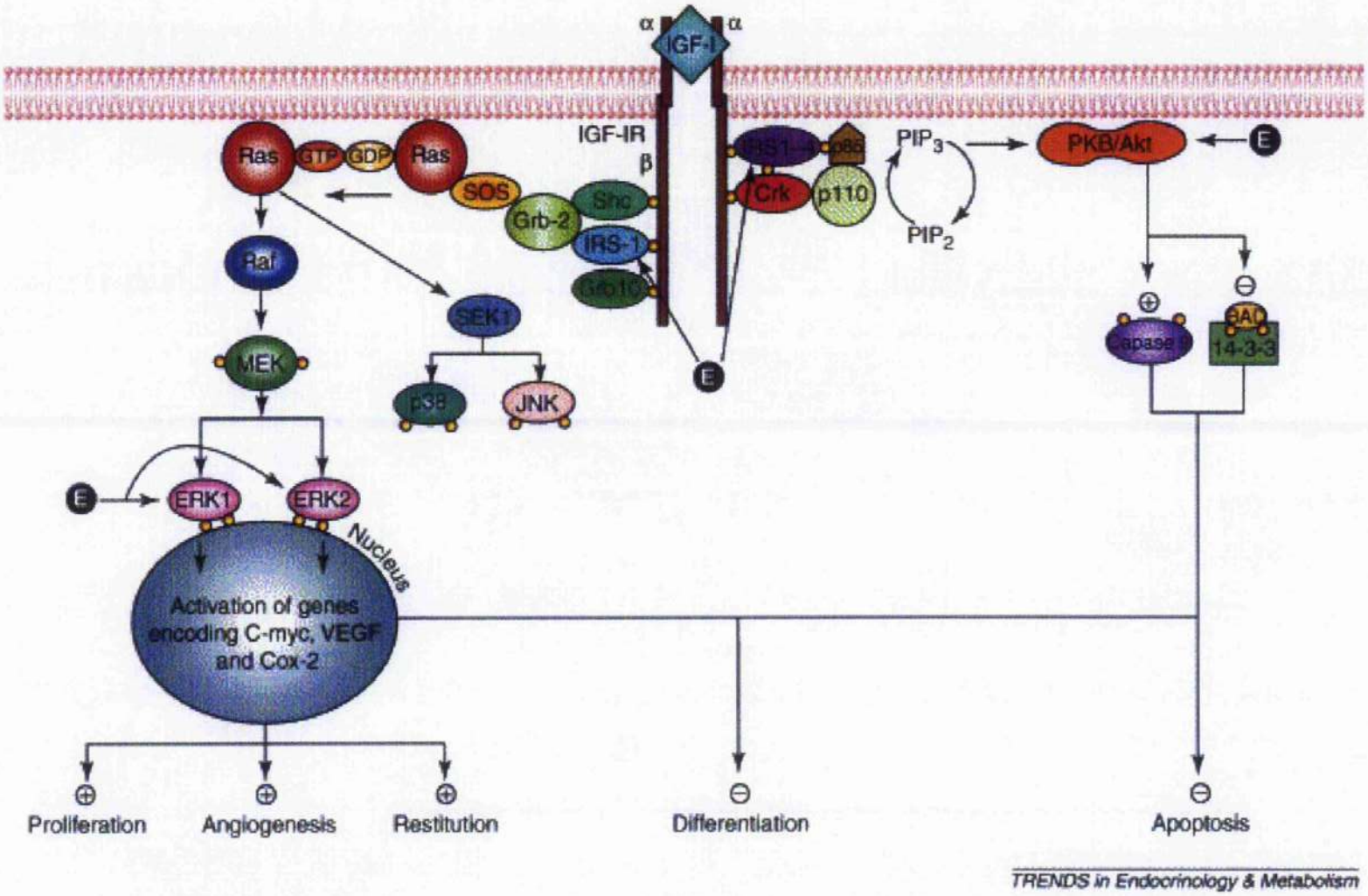


Figure I.3 IGFs/IGF-IR signal pathway (Laban et al., 2003).

I.1.3 Insulin-like growth factor binding proteins (IGFBPs): General protein structure

Analysis of the amino acid sequence reveals that the IGFBPs are clearly distinct but share regions containing strong homology. The structure of IGFBPs is generally described as consisting of three domains of approximately equal size: the conserved amino- (N-) terminal domain, the highly variable midregion and the conserved Carboxyl- (C-) terminal domain. IGFBPs share at least 50 % homology among themselves, and 80 % homology between different species (Lamson et al., 1991; Shimasaki et al., 1991). The mature proteins contain between 216 and 286 amino acids, giving core molecular masses of between 22.8 and 31.3 kDa.

The IGFBPs are cysteine rich proteins: 16-20 cysteines in the pre-peptides (prior to removal of the signal peptide, which is necessary for secretion). Structurally, the cysteines are clustered at the conserved N terminal domain (12 cysteines in IGFBP-1, -2, -3, -4 and -5, whereas there are only 10 in IGFBP-6) and at the conserved C-terminal third (6 cysteines) of the proteins. The alignment of the cysteine molecules is strongly conserved across all the six IGFBPs (Shimasaki et al., 1991). The higher number of cysteine residues in these domains suggests that this part of the proteins is likely to be highly structured. It is believed all of the cysteines are engaged in disulphide bridges, and disulphide linkages of cysteines are important for the correct folding and maintenance of the three-dimensional structure of many proteins. In addition, it has been shown that the N-terminal cysteines in bovine IGFBP-2 form sequential pair-wise disulphide bridges, which demonstrates that there are no inter-domain disulphide linkages between cysteines in the N- and C-terminal domains (Forbes et al., 1998). In contrast to the N- and C-terminus, the central domain has no cysteines except IGFBP-4 (Drop et al., 1992; Hwa et al., 1999). However, this region contains phosphorylation, glycosylation, and many proteolytic cleavage sites suggesting that important biological functions of IGFBPs may be regulated by this region (Conover, 1995).

I.1.3.1 IGF binding motifs

One of the key features of the IGFBPs is their unique ability to bind IGFs with high affinity (Ballard et al., 1989; Baxter, 2000). To date, the structure of IGFBP proteins has not as yet been determined by X-ray crystallography and it has been argued that this may be due to several factors ranging from the difficulty in expressing recombinant IGFBPs at high enough concentrations to the possibly flexible nature of the midregion part of these proteins, which would make crystal formation problematic. However, based on two-dimensional nuclear magnetic resonance (NMR) spectroscopy using IGFBP fragments (Kalus et al., 1998) and mutagenesis (Forbes et al., 1998; Imai et al., 2000) studies, it is believed that IGFBP proteins make contact with the IGFs with their N- and C-terminal domain simultaneously. From this work it was argued that the cysteine-rich N- and C-terminal ends are brought into alignment to create a high-affinity IGF binding site by bending of the "flexible" central domain of the IGFBPs (reviewed in Clemmons, 2001).

The observation that the N- terminal fragment of IGFBP-3 was capable of binding IGF-I and -II, although with relatively low affinity (Durham et al., 1997; Fowlkes et al., 1995), and the C-terminal domain of IGFBP-2 alone was shown to retain some binding activity (Wang et al., 1993) initially suggested that important binding sites existed within both the N- and C-terminal domains. This suggestion was subsequently supported by the demonstration that both N- and C-terminal regions of IGFBP-3, and the C-terminal fragment of IGFBP-2 had some IGF binding activity (Ho and Baxter, 1997; Spencer and Chan, 1995). Several investigators have begun to identify the important binding site in the N-terminal domain of IGFBPs (Andress et al., 1993; Chernausek et al., 1995; Durham et al., 1997; Hashimoto et al., 1997; Spencer and Chan, 1995; Zapf et al., 1990). Several residues in this region have side chains that could potentially interact with the appropriate side chains of residues in IGF-I or -II that are known to be important for binding IGFBPs (reviewed in (Clemmons, 2001). Some groups have proposed that the N- terminal region contains the major IGF binding site that is common to all six IGFBPs and further speculated that this region interacts with an important binding domain in the C-terminus of each protein to establish the high affinity-binding site (Hashimoto et al., 1997; Kalus et al., 1998; Qin et al., 1997). Preliminary findings

showed that the affinity of fragments containing this N-terminal domain-binding subunit was relatively low, and this led to the speculation that although the N-terminal region is absolutely required for IGF binding, the C-terminal domain is also necessary to stabilise the IGF/IGFBP complex (Hashimoto et al., 1997; Kalus et al., 1998; Qin et al., 1997).

In support of this finding, it was shown that the basal binding subunit for the IGFs was contained in the sequence encompassing the Glu 52 to Ala 92 of rat IGFBP-3 using a solid-phase binding assay (Hashimoto et al., 1997). The authors were also to show that this fragment has IGF binding affinity, although it only constitutes a small part of the native protein. These researchers also showed that the affinity for IGF-I was decreased 25-fold with a fragment containing residues 1-92. However, when the middle portion (between 1-186) was included, the affinity was only reduced 8-fold. Therefore, it was concluded that flanking sequences in both the N-terminal and C-terminal regions adjacent to this sequence is important for folding (reviewed in Clemmons, 2001).

Using NMR spectroscopy, it has been shown that IGFBP-5 is nearly identical in sequence to IGFBP-3 between residues 52-92 (Kalus et al., 1998). In this study, a fragment of IGFBP-5 containing residues 1-94 was prepared by limited proteolytic digestion of the whole protein, as well as a C-terminal fragment (residues 135-246) and a midregion fragment (residues 95-134). An additional fragment containing the sequence between Ala 40 and Glu 92 was also generated by this proteolytic digest. BIAcore analysis was used to determine the binding affinities of each of the fragments for IGF-I and IGF-II. Neither the C-terminal fragment nor the midregion fragment had detectable affinity. In contrast, the N-terminal fragment and the smaller 40-92 fragment had easily detectable affinities, albeit that they were reduced 200-fold compared with full-length IGFBP-5. Although the association rate of IGF-I binding to the 40-92 fragment approximated that of the native protein, its binding to this peptide was associated with a much more rapid dissociation rate. Furthermore, the affinity of the Ala 40-Glu 92 fragment was sufficient to inhibit IGF-II-stimulated phosphorylation of the IGF-I receptor. From these data, it was possible to construct a three-dimensional model of the region contained within the mini IGFBP-5 sequence that is shown in Figure I. 4. This structure comprises a uniquely folded domain containing three anti-parallel β-strands and two disulphide bridges that are responsible for its compact structure. This structure is

further strengthened when IGF-I or -II binds to the peptide. These cysteine pairings are also present in IGFBP-2, -3 and -6 (Hashimoto et al., 1997; Neumann and Bach, 1999). Since there was no significant difference in affinity between the larger 1-92 N-terminal fragment and the smaller 40-92 fragments, it was concluded that this contained the major IGF binding site. However, it was also maintained that in order to obtain the full affinity of the native protein, this requires complex formation with C-terminal domain residues resulting in stabilisation of the intact protein (Clemmons, 2001).

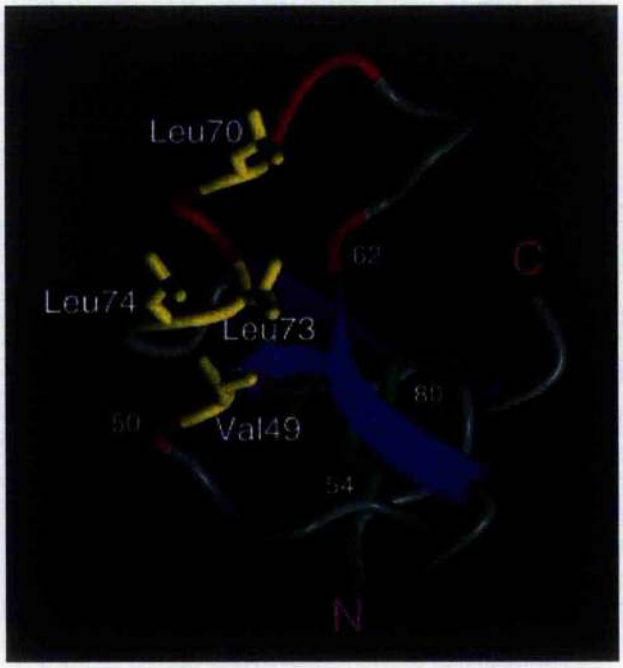


Figure I. 4 Tertiary structure of the mini-IGFBP-5. Ribbon drawing of mini-IGFBP-5 showing its β-sheet, the cysteine bridges and the residues composing the hydrophobic patch. The charged and hydrophobic amino acids are believed to form the high-affinity binding pocket in the N-terminal region of IGFBP-5. These amino acids have been proposed to interact with amino acids containing specific side chains and charged groups within IGF-I and IGF-II (Kalus et al., 1998).

In the N-terminal major IGF-binding site model, in solution the residues Val 49, Leu 70, and Leu 74, expose their hydrophobic side chains in a specific alignment that defines the hydrophobic patch on the surface of this IGFBP-5 fragment. In addition, Val 49, Leu 73, and Leu 74 of IGFBP-5 are conserved among all IGFBPs, suggesting that this model is likely to be relevant for the binding of the other IGFBPs to IGF-I or IGF-II (Kalus et al., 1998). It is further supported by site directed mutagenesis studies of IGF-I and IGF-II (Bach et al., 1993; Francis et al., 1993). The IGF-II residues known to bind IGFBPs are Phe 48 and Glu 6, which could bind to Leu 73/Leu 74 and Lys 68 of mini IGFBP-5 (residues Ala 40 to Glu 92) respectively. However, the sequence between positions 62 and 69 in IGFBP-5 is less well conserved amongst the other IGFBPs, and this could explain the variation in affinity for IGF-I and -II observed amongst the six binding proteins. This is further supported by the observation that this region is conserved in IGFBP-3, which has an affinity for IGF-I and -II that is similar to that of IGFBP-5. Furthermore, a basic residue which is conserved between IGFBP-3, -4, and -5 (position 68 in IGFBP-5) (Lalou et al., 1996), appears to be necessary for the highest affinity (reviewed in Clemmons, 2001).

A further *in vitro* mutagenesis study (Imai et al., 2000; Kalus et al., 1998) confirmed the model of Kalus and colleagues, suggesting that these residues in the N-terminus of IGFBP-3 and -5 are required for the formation of a high-affinity IGF-I binding pocket in the intact protein (Clemmons, 2001).

Less work has focussed on the C-terminal domain of the IGFBP proteins with respect to IGF binding. However, one group carried out C-terminal truncations in bovine IGFBP-2 (Forbes et al., 1998) and identified a short stretch of residues that, once deleted, resulted in an 80-fold reduction in affinity for binding to IGF-II. Alignment of the amino acid sequence for this region in all six IGFBPs from all species analysed to date demonstrates that there are two completely conserved amino acids, Gly203 and Gln209 (residue numbers for murine IGFBP-5) (Bramani et al., 1999b). The substitution in the C-terminal region of IGFBP-4 was predicted to alter its folding pattern (De la Fontaine et al., 1995). Mini deletion analysis of the region from Cys 205 to Val 214 revealed that this region was critical for optimum affinity, and this deletion resulted in a 6-fold reduction in its affinity. The authors concluded that the N-terminal sequence Leu 72 to Ser 91

contained the basal binding sub unit and the region from Cys 205 to Val 214 was necessary for proper folding and attainment of high-affinity binding.

Our group has previously presented evidence that the C-terminal domain of IGFBP-5 is necessary for optimal IGF-I binding (Bramani et al., 1999b; Song et al., 2000). These researchers determined that two specific amino acids in the C-terminal region of IGFBP-5 were critical for maintenance of high affinity binding using site directed mutagenesis to mutate Gly 203 to Lys and Gln 209 to Ala. This caused an 8-fold reduction in affinity of IGFBP-5 for human IGF-I. Since this region of IGFBP-5 has also been shown to be very important for ECM binding, the authors hypothesised that when the IGFBP-5 binds to ECM, its affinity for IGF-I is reduced. Based on the data of Kalus and co-workers (Kalus et al., 1998) showing that the C-terminal fragment has no intrinsic binding activity and that this sequence may be responsible for forming the high affinity tertiary structure, this suggested that these substitutions resulted in a major alteration in its alignment with the N-terminal region-binding site, thus disrupting the affinity of the whole protein. Conversely, substitutions for Gly 203 and Gln 209 resulted in no change in the affinity of this form of IGFBP-5 for heparin. Since binding of IGFBP-5 to ECM or heparin had been shown to lower the affinity of IGFBP-5 for IGF-I, this suggests that the heparin/ECM binding domain and the IGF-binding domain overlap. The authors further proposed that the ability of heparin binding to alter the affinity of IGFBP-5 (Arai et al., 1994) for IGF-I is due, at least in part, to steric hindrance (Song et al., 2000).

These conclusions were supported by a second study, which demonstrated that substitutions for five amino acids in IGFBP-3 (K228-R232) with residues from the corresponding sequence in IGFBP-1 resulted in a substantial reduction (20-fold) in the affinity of IGFBP-3 for IGFs (Firth et al., 1998b). These residues are homologous to the R214-R218 sequence in IGFBP-5. They further showed that deletion of IGFBP-3 residues from 185-264 or from 89-184 markedly reduced IGF-I and -II binding. However, some affinity of the 1-89 fragment was retained, consistent with the results of others that the N-terminus contains an important binding site (Kalus et al., 1998).

Therefore, the observation that residues involved in IGF binding occur in both N- and C-terminal domains implies the existence of an IGF-binding pocket involving both domains as shown in Figure I. 3 (Diagram of IGFBP structure from (Firth and Baxter, 2002). In this figure, other important functional subdomains and sites of posttranslational modification are also indicated and will be discussed later.

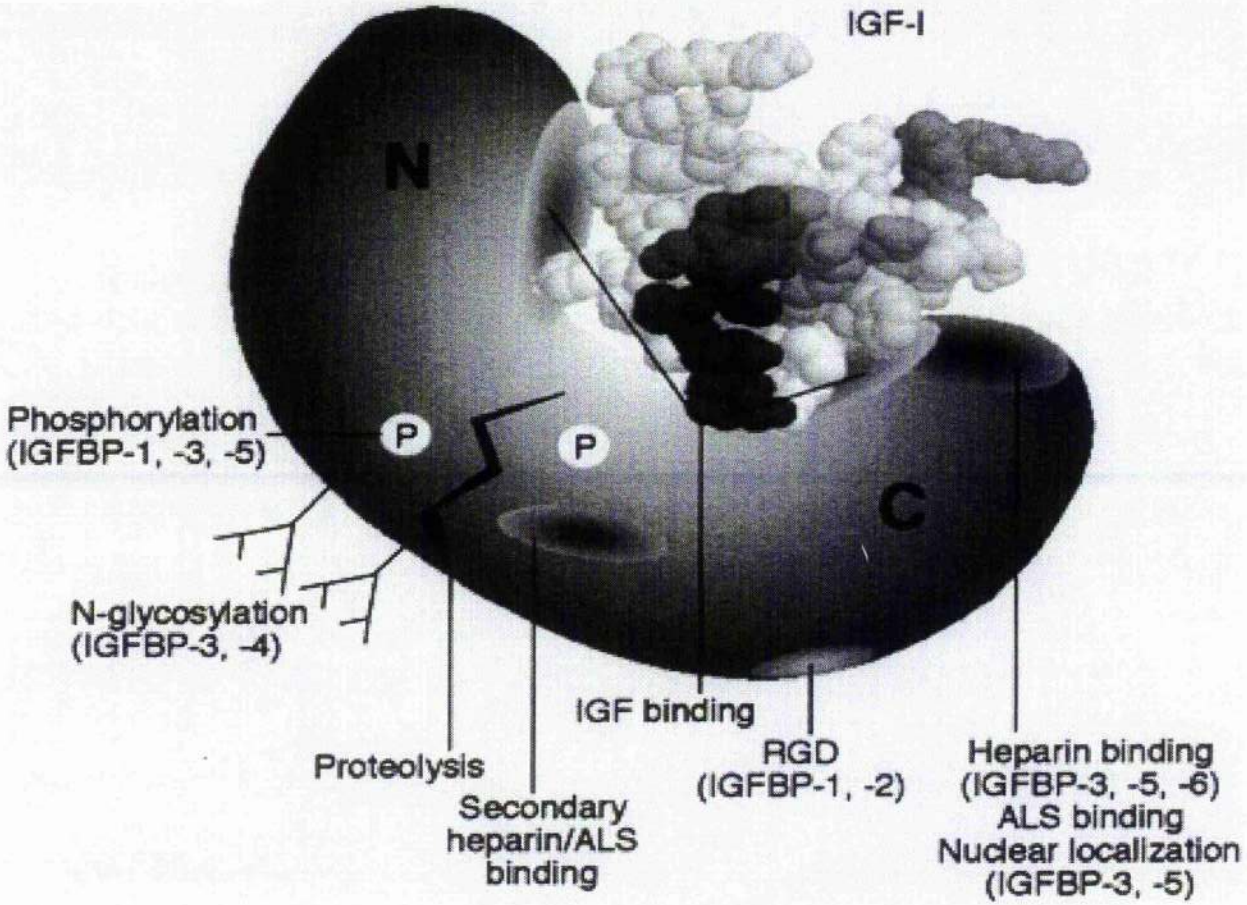


Figure I. 5 Generalized diagram of IGFBP structure showing proposed interaction with IGF-I through both N and C domains. Functional domains and sites of posttranslational modification are indicated (Firth and Baxter, 2002).

I.1.3.2 Cell membrane and ECM binding motifs

Some IGFBPs not only bind IGFs, but also interact with the surface of cells or to extracellular matrix (ECM), and various cell-association molecules or putative receptors (reviewed in (Firth and Baxter, 2002). As shown in Figure I.4 (diagram of IGFBP structure), important subdomains have been identified within the C-terminal region of various IGFBPs; for example, Arg-Gly-Asp (RGD) integrin-binding motifs are located at residues 221-223 of IGFBP-1 (Jones et al., 1993d) and residues 265-267 of IGFBP-2 (Binkert et al., 1989) and these have been shown to facilitate binding of these two IGFBPs to the $\alpha_5\beta_1$ integrin receptor (Jones et al., 1993d; Rauschnabel et al., 1999; Schutt et al., 2004). Functionally important 18-residue basic motifs with heparin-binding activity have also been identified at residues 215-232 of IGFBP-3 and residues 201-218 of IGFBP-5 and are also involved in the interaction with the serum glycoprotein ALS (Firth et al., 2001; Firth et al., 1998b; Twigg et al., 1998), plaminogen activator inhibitor-1 (PAI-1) (Nam et al., 1997), transferrin (Weinzimer et al., 2001) and proteins involved in cell and matrix binding (Booth et al., 1995; Firth et al., 1998b) and nuclear transport (Schedlich et al., 2000), discussed in detail later. There are numerous reports that the cell association of IGFBP-3 and -5 could lead to both potentiation and inhibition of IGF actions (Booth et al., 1995; Conover, 1992; Lalou et al., 1996; Martin et al., 1992).

ECM binding motif Biochemical analysis has shown that IGFBP-5 is the most abundant form of IGFBP in the ECM derived from connective tissue cells (reviewed in Clemmons, 2001), whereas there are minimal concentrations of IGFBP-3 present in ECM from fibroblast cells (Jones et al., 1993c). This group also found that adding increasing concentrations of salt resulted in the decreased binding of IGFBP-5 to fibroblast ECM. This clearly demonstrated that the binding of IGFBPs to the ECM 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.

IGFBP-3 and -5 have the greatest potential to bind ECM via a stretch of basic amino acid in their C-terminal domain. This sequence is highly conserved between IGFBP-3 and -5, because these two IGFBP genes are thought to originally have been duplicated from a single gene (Allander

et al., 1995). However, in spite of this sequence similarity, the major differences in the amounts of ECM-bound IGFBP-3 and IGFBP-5 perhaps suggests that the region of IGFBP-5 that accounts for ECM binding is folded differently in IGFBP-3 such that there is less surface exposure of this sequence (Clemmons, 2001).

Several studies have focused on the ability of two highly basic regions within IGFBP-5 to bind to the ECM. One sequence is located between amino acids 131 and 141 in the central domain, and the other between 201 and 218 in the C-terminal domain. Small oligopeptides were prepared that contained the sequences from each of these regions, and their ability to inhibit IGFBP-5 binding to human fibroblast ECM was determined. Although the 131-141 region contained a higher percentage of basic residues, it accounted for very little ECM binding, and the principal ECM-binding site was located in the 201-218 region (Jones et al., 1993c; Rees and Clemmons, 1998).

To further determine the important amino acids in IGFBP-5 for ECM binding, several mutagenesis studies were conducted. IGFBP-5 mutants, engineered to contain up to four neutral substitutions for a combination of the ten basic residues between 201 and 218, were synthesized and expressed and their ability to bind ECM was compared to the native IGFBP-5 protein. A systematic mutational analysis of this region using, either single or combined substitutions of basic amino acids, identified Arg 207 and Arg 214 as the most critical amino acids for ECM binding (Parker et al., 1998).

Furthermore, binding studies show that IGF has decreased affinity for IGFBPs when the IGFBP is associated with the cell surface or matrix (reviewed in Baxter, 2000)). For IGFBP-3, a 40-fold lower IGF-I affinity was reported when IGFBP-3 was bound to the human fibroblast cell lines GM10 and T98G compared with IGFBP-3 in solution (McCusker and Clemmons, 1997); IGFBP-2, which associates with cell-surface proteoglycans in the brain, shows a 3-fold decrease in IGF affinity when bound to chondroitin sulfate (Russo et al., 1997). In the case of IGFBP-5, binding to fibroblast matrix decreases its affinity for IGF I 7-fold (Jones et al., 1993c).

A helical wheel prediction of the 201-218 residues of IGFBP-5 (Figure 1.6) places the critical ECM binding residues, Arg 207 and Arg 214, adjacent to Gly 203, which was demonstrated to be critical for IGF binding, while Glu 209, another amino acid essential for IGF binding, is flanked by two other basic residues in the wheel (Bramani et al., 1999b; Parker et al., 1998). In addition, mutation of the basic residues 201, 202, 206 and 214 resulted in attenuated heparin binding, but only a small reduction in the affinity for IGFs (Song et al., 2000). This strongly suggests that ECM and IGF binding sites are located in close proximity to each other and may even overlap, providing an alternative explanation for the reduced IGF affinity of ECM-bound IGFBP-5 (Bramani et al., 1999b; Schneider et al., 2002), rather than binding of heparin to this domain producing a conformational change that might reduce the affinity for the IGFs (Andress and Birnbaum, 1992; Arai et al., 1994; Jones et al., 1993b).

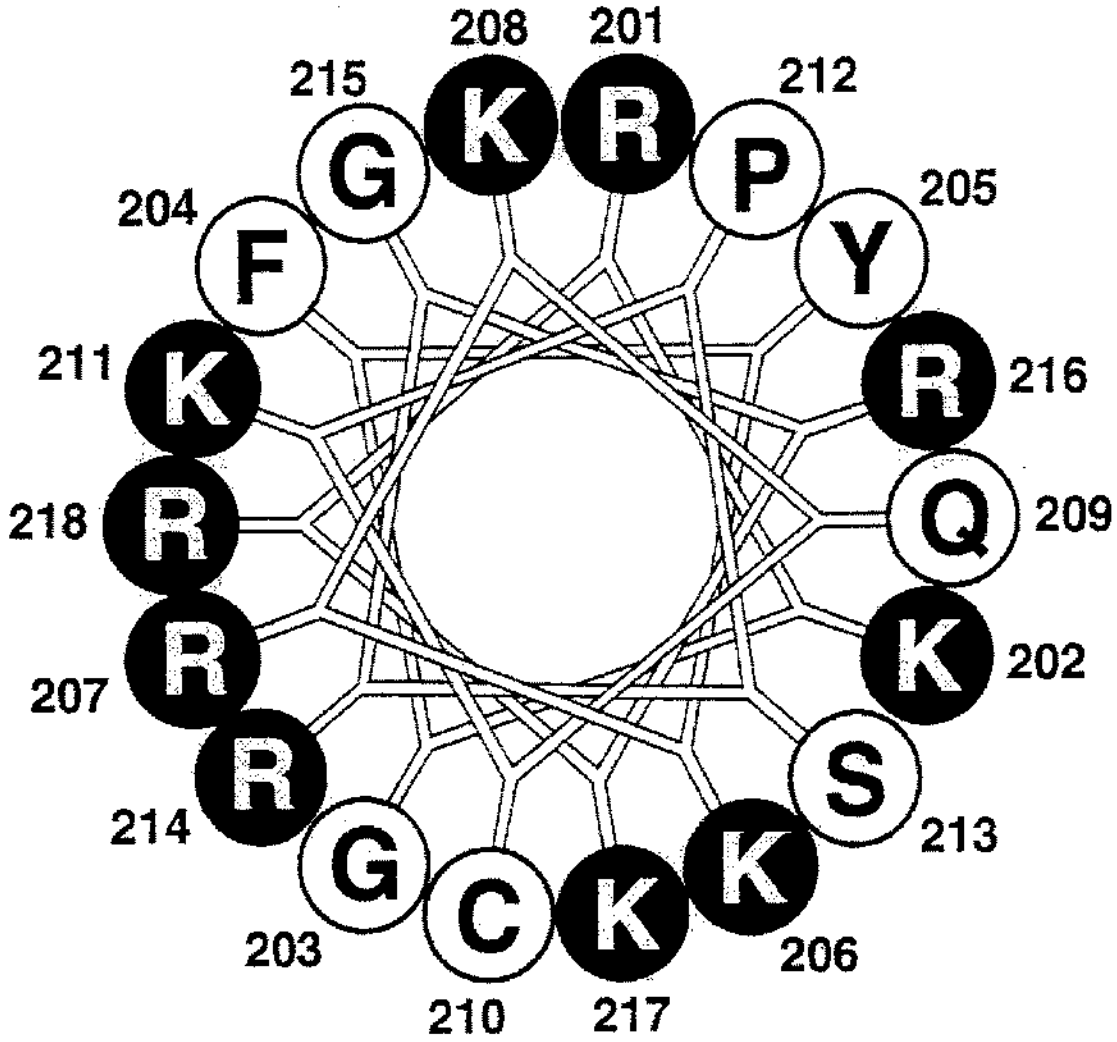


Figure I. 6 Helical wheel alignment of 201-218 region of IGFBP-5.

Arg-Gly-Asp (RGD) integrin binding motif Cell biological events such as the interaction between cells and extra cellular matrix are mediated by cell surface integrin receptors. Integrins modulate cell proliferation and apoptosis, as well as mediating cell attachment, migration and spreading. These receptors are made up of an α and a β subunit. Integrins have no intrinsic enzyme activity, but their activation can lead to the recruitment of signalling proteins which form focal adhesion complexes. From the literature, it has been shown that there are at least 50 different proteins, including focal adhesion kinase (FAK) and paxillin, that associate at sites of focal adhesions and transduce signals that mediate changes in cell shape or gene expression (Berditchevski, 2001; Cary and Guan, 1999; Clark and Brugge, 1995; Clezardin, 1998; Zamir and Geiger, 2001).

Particular integrin receptors recognise specific ECM components such as fibronectin and vitronectin. Many ECM proteins, such as fibronectin, contain an amino-acid sequence, RGD, which is a consensus recognition sequence for the integrin receptor family and which, ultimately, modulates cell attachment and motility. The RGD integrin binding motif has been identified in the C-terminus of IGFBP-1 and -2, but in no other IGFBP (Jones and Clemmons, 1995).

The RGD sequence in IGFBP-1 has previously been shown to be functional. IGFBP-1 was shown to stimulate Chinese hamster ovary (CHO) cell migration in an IGF-independent manner, the effect being mediated through its RGD sequence via the $\alpha_5\beta_1$-integrin receptor (fibronectin receptor) (Jones et al., 1993d). Similarly, $\alpha_5\beta_1$ integrin binding by IGFBP-1 was found to be important in human trophoblast cell migration, because antibodies against either integrin subunit blocked the stimulatory effect of IGFBP-1 (Irving and Lala, 1995). The RGD motif in IGFBP-1 also appears to be involved in the induction of FAK dephosphorylation, cell detachment, and subsequent apoptosis, because addition of IGFBP-1 to T47D and Hs578T breast cancer cells which have $\alpha_5\beta_1$ integrin receptor promoted the dephosphorylation of FAK (Perks et al., 1999b). In addition, IGFBP-2 has shown to bind breast cancer cells and Ewing sarcoma cells via the $\alpha_5\beta_1$ integrin receptor (Schutt et al., 2004; Shutt et al., 2000), and these authors also reported an associated decrease in FAK phosphorylation.

GAGs or heparin binding motif Most of the IGFBPs have cell surface glycosaminoglycan (GAG: such as heparin and heparan sulphate proteoglycan) binding properties (Hodgkinson et al., 1994). GAGs are normal components of the cell surface and extracellular matrix and one particular GAG, heparin, is produced by most cells and stored in cytoplasmic granules, before being released into the circulation during inflammatory processes. Heparin can easily compete for binding of IGFBPs to cell surface-associated binding sites and is believed to be involved in the translocation of IGFs to extra-vascular tissues (Andress, 1995; Andress, 1998; Booth et al., 1995; Fowlkes and Serra, 1996).

The importance of GAG binding for IGFBPs has been demonstrated by the fact that this can result in the dissociation of the components of the IGF/IGFBP-3/ALS complex in serum (Baxter, 1990). It has also been shown that IGFs can localize to specific cell types that do not express IGF mRNA, but which synthesize IGFBPs, and it was postulated that ECM/cell surface-associated IGFBPs could direct IGF to specific cell types within the tissue (Jones et al., 1993c).

Subsequently, it was shown that GAGs could also interfere with both the binding of IGFBP-3 to the cell surface and of IGFBP-5 to the ECM (Andress, 1995; Booth et al., 1995; Jones et al., 1993c). Furthermore, GAG and proteoglycan binding of IGFBP-3 and -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. It was shown that adherence to proteoglycan results in a major reduction of the affinity of IGFBP-5 for IGF-I by 8-fold, whereas it had only a small 3-fold effect on the affinity of IGFBP-3 (Arai et al., 1994; Jones et al., 1993c). However, the heparin binding capabilities of other IGFBPs has not, as yet, been reported, with the exception that the ability of IGFBP-2 to bind GAGs requires the binding protein to be pre-complexed with either IGF-I or -II (Arai et al., 1996a).

The specific amino acids in IGFBP-3 and IGFBP-5 that bind to cell surface proteoglycans have not been definitively identified. However, several studies have shown that a peptide containing amino acids 201-218 of IGFBP-5 competitively inhibited IGFBP-5 binding to cell surface proteins and to proteoglycans (Andress, 1995; Jones et al., 1993c; Rees and Clemmons, 1998). Using a combination of substitutions for charged amino acids in the 201-218 region of

IGFBP-5, it was deduced that the heparin binding motif, K206 R207 K208 Q209 C210 K211 (BBBXXB, where B represents a basic amino acid and X is any residue), contained the primary binding site (Arai et al., 1994). Mutants with substitutions for several of the amino acids in this region demonstrated that R201, K202, K206 and R214 were required for heparin binding, while not affecting IGF binding (Arai et al., 1996b; Song et al., 2000). For IGFBP-3, the corresponding sequence lies between amino acids 214 and 232, which also contains a heparin-binding consensus site (Booth et al., 1996; Campbell and Andress, 1997a; Fowlkes and Serra, 1996). A definitive study published by Firth and colleagues (Firth et al., 1998b) in which they used a mutant form of IGFBP-3, where the residues from K228 through R232 had been substituted with the corresponding residues from IGFBP-1, demonstrated that the mutant protein associated much less with cell surfaces, strongly suggesting that these residues were an important component of the cell surface binding site.

Heparin binding to IGFBP-5 results in an 8- to 12-fold reduction in its affinity for IGF-I (Arai et al., 1996a). When the effects of specific amino acid substitutions on this affinity shift were determined, it was noted that single substitution for K211 or a double substitution for K217 and R218 resulted in no reduction in heparin binding, but that these substitutions were associated with a marked reduction in the ability of heparin to alter the affinity of IGFBP-5 for IGF-I (Arai et al., 1996b). As noted for ECM binding, the amino acids Gly 209 and Gln 203 in IGFBP-5 that are important for formation of the high-affinity IGF-binding pocket (Bramani et al., 1999b; Shand et al., 2003), and G203 lies close to these charged residues in a helical wheel model for this region (Figure I. 6). Heparin/ECM bound IGFBP-5 has between an 8- to a 17-fold reduced affinity for binding IGF-I (Andress and Birnbaum, 1992; Arai et al., 1994; Jones et al., 1993c). As argued above, this could possibly imply that heparin binding induces a conformational change in IGFBP-5 protein structure or that the heparin binding site may actually physically overlap with an IGF binding site in the C-terminal domain.

I.1.3.3 Post-translational modification

IGFBPs are post-translationally modified by processes including glycosylation and phosphorylation. Most post-translational modifications occur in the central domain, but not in the N- or C- terminal domains (Firth and Baxter, 2002).

Glycosylation There has been no clear evidence to date that IGFBP-1 or -2 are glycosylated, whereas IGFBP-3 and -4 are N-glycosylated, and IGFBP-5 and -6 are O-glycosylated (Conover and Kiefer, 1993; Standlker et al., 1998). N- glycosylation occurs 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 are found at Asn 89, Asn 109 and Asn 172 (Zapf et al., 1988) and variability in glycosylation accounts for the 40-50 kDa forms of IGFBP-3 seen on immunoblotting (Firth and Baxter, 1999). IGFBP-4 is N-glycosylated in its central domain on Asn104 (Ceda et al., 1991) and the N-glycosylated and non-glycosylated forms migrate with apparent molecular masses of 28 and 24 kDa respectively. Although there is one potential N-glycosylation site in the C-terminal domain of IGFBP-6, this site does not appear to be glycosylated (Bach et al., 1992). In contrast to N-glycosylation, no amino acid consensus sequence exists for O-glycosylation, therefore the location of these sites must be determined empirically. Even though IGFBP-5 and -6 are both O-glycosylated, only recently were the O-glycosylation sites determined in the central domain of IGFBP-6 at Thr 102, Ser 120, Thr 121, Thr 122, and Ser 128 (Neumann et al., 1998). To date, an exhaustive study of IGFBP-5 glycosylation sites has not been performed, but it has been shown that Thr 152 is O-glycosylated (Standlker et al., 1998).

Although N- or O-linked glycosylation may affect some functions of IGFBPs, such as resistance to proteolysis, it does not appear to affect their ability to bind to IGFs (Neumann et al., 1998). Glycosylation has no effect on high affinity IGF binding by IGFBP-3 (Firth and Baxter, 1999), IGFBP-4 (Chelius et al., 2001), IGFBP-5 (Shand et al 2003) or IGFBP-6 (Bach et al., 1992; Marinaro et al., 2000). For example, comparison of bacterially derived and mammalian CHO cell derived IGFBP-3 indicates that glycosylation has no significant effect on the binding of IGF-I

(Sommer et al., 1993) and also bacterially expressed IGFBP-5 has almost exactly the same affinity for binding IGF-I in solution phase assays and biosensor analysis as commercial mouse *wt* IGFBP-5 protein which was expressed in mammalian cells (Shand et al., 2003), showing that glycosylation of IGFBP-5 has no effect on IGF binding. In addition, N-glycosylation of IGFBP-3 had no effect on ternary complex formation with the acid-labile subunit (Firth and Baxter, 1999). However, IGFBP-3 forms in which various N-glycosylation sites have been altered by mutagenesis reveal that decreasing glycosylation tends to increase cell surface association, so that non-glycosylated IGFBP-3 shows approximately 3-fold higher binding to both CHO cells and T47/D breast cancer cells compared with the fully glycosylated protein (Firth and Baxter, 1999; Firth et al., 1999). Similarly, O-linked glycosylation of IGFBP-6 inhibits cell surface binding, probably by inhibiting binding to glycosaminoglycans (Marinaro et al., 2000). Finally, O-glycosylation of IGFBP-6 also inhibits proteolysis of the binding protein (Marinaro et al., 2000; Neumann et al., 1998).

Phosphorylation Three of the six IGFBPs, IGFBP-1, -3, and -5, have been shown to be post-translationally phosphorylated on Ser residues (Coverley and Baxter, 1997; Jones et al., 1991). Human IGFBP-1 might be phosphorylated on Ser 101, Ser 109 (both in the central domain) and Ser 169 (in the C-domain), with Ser 101 being the predominant phosphorylation site (Jones et al., 1993a), whereas rat IGFBP-1 might be phosphorylated on Ser 70 and Ser 132 in the central domain (Peterkofsky et al., 1998). Human IGFBP-3 might be phosphorylated on Ser 111 and Ser 113 in the central domain (Hoeck and Mukku, 1994), whereas phosphorylation of IGFBP-5 has only been reported in one abstract and the site was not proven (Jones et al., 1992; reviewed in Schneider et al., 2002).

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 signal transduction pathways, the cell cycle, and 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 IGF-I 6-fold (Jones et al., 1991), but has no effect on its affinity for IGF-II (Jones et al.,

1991). Phosphorylation of rat IGFBP-1 (Peterkofsky et al., 1998) and human IGFBP-3 (Hoeck and Mukku, 1994) has no effect on IGF binding. There is also evidence that phosphorylation inhibits IGFBP-3 cell surface association (Coverley et al., 2000). However, although the biological significance of IGFBP-5 phosphorylation has not been elucidated, it is clear that, as with glycosylation above, the affinity for phosphorylated and non-phosphorylated IGFBP-5 for IGFs is the same, as evidenced by comparing bacterially expressed protein and that expressed in mammalian cells (Shand et al., 2003).

Proteolytic cleavage All IGFBPs can be cleaved by spedific proteases at lease 3 classes: serine protease, metalloproteases and aspartic proteases (Parker et al., 1995) and this results in reduction or loss of IGF-binding activity (Firth and Baxter, 2002). Most cleavage sites for IGFBPs are in their central domain which non-conserved region suggesting a potential mechanism by which IGF activies can be regulated in a tissue-specific mannor (Clemmons, 1997) and this is possibly because this region is more surface-exposed (Fowlkes et al., 1997).

IGFBP-3 and -5 are known to cleaved by plasmin (Campbell and Andress, 1997b). Plasminogen, a precursor of active plasmin, binds to the highly basic C-domain regions of IGFBP-3 (Campbell et al., 1999) and IGFBP-5 (Campbell and Andress, 1997b). Similarly, the same region of IGFBP-3 is involved in binding fibrin and fibrinogen (Campbell et al., 1999). Binding of protease precursors to IGFBPs may be a mechnism whereby proteolysis of specific IGFBPs is achieved. Further, GAGs inhibit proteolysis of IGFBP-5 and a possible mechanism is via inhibition of binding of protease precursors (Campbell and Andress, 1997a). Similarly, a potential mechnism whereby ALS prolons the circulating half-life of IGFBP-3 and -5 may be inhibition of binding of pretease precursors, since ALS binds to the same regions of IGFBP-3 and -5 (Parker et al., 1998).

I.1.4 Functions of IGFBPs

It has been well established from *in vitro* studies that IGF signalling through the IGF-IR results in acute anabolic effects on metabolism as well as on cell replication and differentiation (Jones and Clemmons, 1995). However, the bioactivity of IGFs is not only dependent on their

interaction with IGF-IR, but is also influenced by the family of IGFBPs in the local cellular environment, which can potentially either inhibit or enhance IGF actions depending on the complement of IGFBPs present (Firth and Baxter, 2002).

I.1.4.1 IGF-dependent functions

The term "IGF-dependent" functions of IGFBPs has been used to define functions of IGFBPs, both positive and negative, that are directly linked with IGF bioactivities (Jones and Clemmons, 1995).

I.1.4.1.1 Inhibition of IGF activity by IGFBPs

As discussed earlier, the diverse activities of IGFs in stimulating mitogenesis, increasing substrate uptake and metabolic activity, inhibiting apoptosis, and modulating a variety of specific functions in highly differentiated cell types are for the most part mediated through binding and activation of IGF-IR (reviewed in Baxter, 2000). Furthermore, many *in vitro* studies have demonstrated that all IGFBPs can have growth-inhibitory effects through binding IGFs, and presumably through prevention of IGF binding to the IGF-IR (Ferry et al., 1999). Studies *in vivo* have also suggested that IGFBPs inhibit growth by reducing the free IGF levels (Hasegawa et al., 1997; Powell et al., 1999). These inhibitory effects of all six of the IGFBPs on IGF action have been shown to operate at all levels from cellular DNA synthesis to blood glucose regulation to whole body growth (Bach et al., 1994; Cheung et al., 1991; Cox et al., 1994; De Mellow and Baxter, 1988; Hoflich et al., 1998; Imai et al., 1997; Lewitt et al., 1991) and have been extensively reviewed (Martin and Baxter, 1999; Murphy, 1998).

Since IGFBPs are well-established to be secreted proteins, the extracellular sequestration of IGFs by IGFBPs has been proposed to result on the consequent loss of interactions between IGFs and IGF-IR (Hwa et al., 1999). Studies using the IGF-I analog des-(1-3)-IGF-I, which binds IGF-IR and stimulates DNA synthesis, but does not bind IGFBPs, supports this sequestration

mechanism. In the human pro-myeloid cell line HL-60, adding IGFBP-3 to serum-free media inhibited cell proliferation induced by IGF-I and IGF-II, but not by des-IGF-I (Li et al., 1997b).

Molecular evidence to support the importance of IGFBP proteolysis on IGF-dependent actions was demonstrated when site specific mutagenesis of a major proteolytic site in the central domain of IGFBP-5 (Imai et al., 1997) and IGFBP-4 (Rees et al., 1998), resulted in enhanced IGFBP growth-inhibitory effects. Furthermore, mutagenesis of the IGFBP-4 cleavage sites demonstrated that a protease-resistant mutant was more active in inhibiting IGF-I action (Conover et al., 1995a). IGFBP fragments generated by cellular endo-proteases typically show a marked loss of IGF binding, and proteolytic degradation of IGFBPs to these low-activity forms appears to be an important mechanism by which cells can regulate IGF activity (Baxter, 2000; Hwa et al., 1999). Thus IGFBP-degrading proteases secreted by prostate and breast cancer cells have been proposed to act as growth stimulators by increasing local IGF availability (Conover et al., 1995b; Salahifar et al., 1997), and the timed release of IGFBPs and their proteases during differentiation of a variety of cell types, including osteoblast cells, suggests that IGFBP degradation could play a key regulatory role (Thrailkill et al., 1995).

In general, phosphorylated IGFBP proteins inhibit IGF-I action, whereas de-phosphorylated forms seem to have some stimulatory activity (Clemmons, 1993). It has been proposed that this effect may be caused by the non-phosphorylated binding protein having an IGF affinity low enough to approximate that of the receptor, whereas upon phosphorylation of the IGFBP there is a several-fold increase in IGF affinity, which results in exclusion from the receptor and an overall inhibitory effect on IGF biological actions. In support of this it has been demonstrated that de-phosphorylated IGFBP-1 has been shown to enhance IGF-I-induced DNA synthesis (Busby et al., 1988; Elgin et al., 1987; Yu et al., 1998), whereas phosphorylated IGFBP-1 inhibits IGF-I effects (Busby et al., 1988; Ritvos et al., 1988; Yu et al., 1998).

One study suggested an alternative mechanism of IGF regulation whereby IGFBP-3 interacts with the IGF-I receptor, causing conformational change in the receptor and subsequent loss of affinity for IGF-I (Mohseni-Zadeh and Binoux, 1997).

I.1.4.1.2 Stimulation of IGF activity by IGFBPs

The stimulation of IGF activity was first described for IGFBP-1 and -3, although it is now also well documented for IGFBP-5 (Baxter, 2000). Although the expression of IGFBP-2 is reported to cause enhanced growth of tumour cells (Menouny et al., 1998), evidence that it can potentiate IGF activity is sparse.

IGFBP-1 enhanced the effect of IGF-I on DNA synthesis several fold in porcine aortic smooth muscle cells and chick embryo fibroblasts (Clemmons and Gardner, 1990; Elgin et al., 1987; Koistinen et al., 1990), and, as discussed above, others have shown that non-phosphorylated IGFBP-1 can stimulate the effects of IGF-I (Jones et al., 1991). Thus, in certain circumstances, IGFBP-1 may prolong IGF/receptor interaction by providing a slow and steady release of IGF-I. This is supported by another study which found that an IGF-I mutant that binds poorly to IGFBP-1 did not produce optional stimulation of DNA synthesis in human fibroblasts compared to a wild type IGF-I (Clemmons et al., 1990).

Potentiation of IGF activity by IGFBP-3 has been demonstrated in many cell culture systems. It has even been shown that pre-incubation of the cells with IGFBP-3 followed by its removal still caused subsequent potentiation of IGF-I effects (Conover, 1992; De Mellow and Baxter, 1988). This study proposed that cell associated IGFBP-3 fragments, which would have an affinity to IGF-I that is lower that that of the IGF-IR, might enhance the presentation of IGF-I to its receptor. IGF-I has also been shown to stimulate release of IGFBP-3, but not IGFBP-4, in a lung cancer cell line (Noll et al., 1996). Intriguingly, it was also demonstrated that in proliferating opossum kidney cells fluorescently labelled IGF-I and IGFBP-3, whether added to the media in combination or alone, were absorbed from the media and co-localised to the nucleus (Li et al., 1997a). Given the presence of a Nuclear localisation signal (NLS) in IGFBP-3, which is absent from IGF-I, and that a non-IGFBP-3-binding analogue of IGF-I is not localized to the nucleus, the authors suggest that IGFBP-3 may carry IGF-I to the cell nucleus, providing another regulatory mechanism for IGF action. In addition, *in vivo* studies have shown that administration of IGFBP-3 in association with IGF-I results in better wound healing and stimulation of growth in rats (Bagi et

al., 1994; Hamon et al., 1993). Finally, over-expression of IGFBP-3 in transgenic mice may enhance the effects of IGF-I, since these animals display visceral tissue enlargement (Neuenschwander et al., 1996).

IGFBP-5 also has been shown to have the potential to potentiate IGF-I activity (reviewed in Baxter, 2000). An important *in vitro* study in fibroblasts demonstrated that IGFBP-5 binds to ECM components including collagen, laminin and fibronectin, causing a loss of IGF affinity but prolonged IGF half-life, thereby potentiating IGF-I stimulatory fibroblast growth when present in the matrix (Jones et al., 1993c; reviewed in Baxter, 2000). Furthermore, administration of recombinant human IGFBP-5 to mice in combination with IGF-I increased serum osteocalcin levels to a greater level than either peptide given alone (Richman et al., 1999). Although stimulatory effects of IGFBPs have been associated with low affinity forms and are hypothesized to involve the stabilization of bound IGFs in binary complexes, or the presentation of IGFs to their receptor by cell- or matrix-bound IGFBPs, no definitive mechanism has yet been explicitly demonstrated for this phenomenon.

I.1.4.2 IGF-independent functions

IGFBPs have been shown to stimulate several biological effects, apart from modulating IGF action, either in the absence of IGFs (IGF-independent effects) or in the presence of IGFs without triggering IGF-IR signalling (IGF-IR-independent effects) (Firth and Baxter, 2002).

One of the first reports of IGF-independent actions of IGFBP-1 was shown on cell motility and adhesion in CHO cell line (Jones et al., 1993d). These authors went on to show that this effect was mediated by the RGD integrin-binding motif present in the carboxyl terminal domain of IGFBP-1 interacting with $\alpha_5\beta_1$ integrin. The RGD motif in IGFBP-1 also appears to be involved in the induction of focal adhesion kinase dephosphorylation, cell detachment, and subsequent apoptosis, because IGFBP-1 and a synthetic RGD containing peptide had similar effects in initiating these events in breast cancer cells (Perks and Holly, 1999; Perks et al., 2002b). Similarly, $\alpha_5\beta_1$ integrin binding by IGFBP-1 was found to be important in human trophoblast cell migration,

since antibodies against either integrin subunit blocked the stimulatory effect of IGFBP-1 (Irving and Lala, 1995).

Although a homologous RGD motif is also present in IGFBP-2, the evidence supporting IGF-independent action of IGFBP-2 mediated by integrin binding is still preliminary (reviewed in Firth and Baxter, 2002). Over-expression of IGFBP-2 in Y-a adrenocortical tumor cells resulted in enhanced proliferation (Hoeflich et al., 2000). This study showed that this proliferative effect of IGFBP-2 over-expression was IGF independent since IGF-IR was down-regulated and an IGF-I analog with decreased IGFBP interaction had the same mitogenic potency as IGF-1. In addition, IGFBP-2 has been shown to be mitogenic for uterine endometrial epithelial cells and osteosarcoma cells in the absence of IGFs (Badinga et al., 1999; Slootweg et al., 1995).

Recently, there has been considerable interest in the ability of IGFBPs, especially IGFBP-3, to induce or modulate apoptosis independently of inhibiting the survival functions of IGF-I (Baxter, 2000; Clemmons, 2001). Mouse IGFBP-3 was shown to inhibit fibroblast growth factor stimulated DNA synthesis, an effect that appeared not to depend on the sequestration of endogenous IGFs, but which was reversed by IGF-I (Villaudy et al., 1991). The addition of IGFBP-3 to human breast cancer cells has been shown to inhibit DNA synthesis independently of its effects on IGF-I actions (Oh et al., 1993a). This study also presented preliminary evidence for an IGFBP-3 receptor. More recently it has been demonstrated that induction of IGFBP-3 expression by transforming growth factor-β (TGF-β) leads to apoptosis through an IGF independent pathway and the TGF-β receptor is also the putative receptor for IGFBP-3 (Leal et al., 1997; Rajah et al., 1997). In addition, proteolysis of IGFBP-3 at the cell surface has been shown to release fragments that have IGF-independent effects that can either enhance or inhibit cellular responsiveness to IGF-I (Lalou et al., 1996; Lalou et al., 1997; Maile et al., 1999; Zadeh and Binoux, 1997). Interestingly, it has also been shown that IGFBP-3 or -5 can enter the cell and bind to several different targets in the cytoplasm (Schedlich et al., 1998). Furthermore, it has been shown that both proteins can localize in the nucleus (Jaques et al., 1997; Schedlich et al., 1998) and bind to important growth-regulatory proteins for cellular differentiation (Liu et al., 2000; Schedlich et al., 1998).

It has also been shown that a 22 kDa proteolytic fragment of IGFBP-5 is able to stimulate directly DNA synthesis in osteoblast cells independently from IGF-I (Andress, 1995). IGF-independent effects of IGFBP-4 and -6 are not well established and it is generally accepted that IGFBP-4 and -6 act primarily through inhibition of IGF actions (Firth and Baxter, 2002).

I.1.5 IGFBP-5 function

IGFBP-5 plays an important role in several biological processes including bone formation, cell death (in the ovary, mammary gland and embryo), kidney physiology and muscle differentiation (Kelley et al., 1996). The actions of IGFBP-5 are complex as it can either inhibit or augment the actions of IGF-I and, in addition, has IGF-independent effects, particularly in relation to apoptosis (reviewed in Baxter, 2000).

I.1.5.1 Bone formation

One group has reported the stimulatory effects of IGFBP-5 on osteoclastic activity and osteoclast formation (Kanatani et al., 2000), in contrast to IGFBP-4, which exerts exclusively inhibitory actions on bone cells both *in vitro* (Mohan et al., 1995b) and *in vivo* (Miyakoshi et al., 1999). These observations demonstrated both IGF-dependent and IGF-independent regulation by IGFBP-5.

The stimulatory actions of IGFBP-5 were postulated to be mediated by its ability to bind to the cell membrane or ECM (Andress and Birnbaum, 1992), since IGFBP-5 has been shown to bind to a component of the ECM of bone (Bautista et al., 1991). The proposed mechanism is that IGFBP-5 sequesters IGFs and concentrates them in bone and then releases the growth factors during bone remodelling or after injury, which would stimulate the proliferation of neighbouring osteoblasts (reviewed in Schneider et al., 2002). The age-related decline in the skeletal concentration of IGF-I and IGFBP-5 supports this model and provides an explanation for the age-related impairment of bone formation versus resorption (Mohan et al., 1995a).

The inhibition of IGFs by IGFBP-5 has also been reported in osteosarcoma cells (Schmid et al., 1995). In contrast to stimulatory actions, which were associated with binding to the ECM (Andress and Birnbaum, 1992; Bautista et al., 1991), the inhibitory activities were present exclusively in the culture medium (Conover and Kiefer, 1993). The important factor responsible for this apparent divergence might be the localisation of IGFBP-5 (Schneider et al., 2002). Similarly, stimulation of growth of human fibroblasts was associated with IGFBP-5 located in the ECM, whereas when present only in the medium, IGFBP-5 was rapidly degraded and did not affect proliferation (Jones et al., 1993c).

I.1.5.2 Cell proliferation and differentiation

In addition to the functions of IGFBP-5 in bone formation, it has been reported that the expression of IGFBP-5 is associated with stimulatory effects on cell proliferation, activation and differentiation (Cheng et al., 1999; Kanatani et al., 2000; Richman et al., 1999; Zimmermann et al., 1997).

The increased expression of IGFBP-5 was demonstrated in myoblast cells during the early stages of cell differentiation (James et al., 1993; Rotwein et al., 1995). Increased IGFBP-5 secretion was also observed in differentiating myoblasts that over-express IGF-II (Stewart et al., 1996) or after treatment with insulin and IGFs (Ewton and Florini, 1995). IGFBP-5 expression has also been reported to increase during cell differentiation in L6A1 muscle cells (Ewton et al., 1998) and in C2 mouse myoblasts (Rousse et al., 1998). In L6A1 cells, IGFBP-5 was shown to have 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). As with the studies on bone cells discussed above, this work also showed that although IGFBP-5 exhibited both inhibitory and stimulatory actions in these cells, IGFBP-4 had only inhibitory actions, inhibiting both IGF-I and IGF-II-stimulated proliferation and differentiation (Ewton et al., 1998).

Other studies examining the role of IGFBP-5 in myoblst differentiation using the mouse myoblast cell line C2, indicated that IGFBP-5 expression increased with the differentiation of this

line (James et al., 1996). However, these authors also showed that the over-expression of IGFBP-5 led to the failure of transfected cells to differentiate normally, whereas antisense RNA expression resulted in a more extensive differentiation of cell cultures (James et al., 1996). Furthermore, these effects could be neutralised by the addition of exogenous IGFs. It has also been demonstrated that IGFBP-5 can block TNF-α-induced apoptosis in differentiating skeletal muscle cells (Meadows et al., 2000).

I.1.5.3 Apoptosis

Increased *in vivo* expression of IGFBP-5 has been observed in tissues undergoing apoptosis, such as the involuting prostate (Nickerson et al., 1998), mammary gland (Tonner et al., 1997), atretic ovarian follicles (Besnard et al., 1996), thyroid (Phillips et al., 1994) and in the rat brain following hypoxic-ischemic injury (Beilharz et al., 1993). As discussed below, it has now been demonstrated that IGFBP-5 is a causative agent in the cell death observed in the involuting mammary gland (Tonner et al., 2002). It has also been reported that IGFBP-5 expression is highly restricted to regions of cell death in the developing mouse limb bud (Allan et al., 2000).

In vitro, endogenous IGFBP-5 has been shown to mediate the growth inhibitory effects of both anti-estrogens (Huynh et al., 1996) and vitamin D-related compounds (Rozen and Pollak, 1999; Rozen et al., 1997) in MCF-7 breast cancer cells. However, the addition of exogenous IGFBP-5 to a different human breast cancer line, Hs578T cells, had the opposite effect by protecting these cells from ceramide-induced apoptosis (Perks et al., 1999a; Perks et al., 2000), suggesting that IGFBP-5 may also have a survival function in response to apoptotic stimuli. However, a later study from the same group showed that a non-IGF binding IGFBP-5 mutant had no effet on ceramide-induced apoptosis in IGF-responsive MCF-7cells, whereas the same mutant blocked IGF-induced survival when it could not bind to IGF. Therefore, Hs578T cells are IGF-non-responsive and so the survival effect of IGFBP-5 in this cell line was IGF-independent (Perks et al., 2002a). These authors suggested that, as this IGFBP-5 mutant could not bind to IGF-I, IGFBP-5 may also be able to block the survival effects of IGF-I via integrin receptors (Perks et al., 2002a).

Although it has previously been demonstrated that IGFBP-3 can translocate to the cell nucleus (Schedlich et al., 1998) and transcriptionally down-regulate the anti-apoptotic Bcl-2 gene (Butt et al., 2000), it was subsequently shown that its nuclear translocation is not necessary for its pro-apototic and anti-proliferative effects in breast cancer cells (Butt et al., 2002). Using a transient over-expression system, IGFBP-5 was also observed in the nuclear compartment of human breast cancer cells (Butt et al., 2003) and was shown to significantly inhibit the growth of these cells both *in vitro* and *in vivo*. In this study, IGFBP-5 expression resulted in an up-regulation of the mRNA for the pro-apoptotic regulator Bax and a down-regulation of the anti-apoptotic Bcl-2. Furthermore, these effects appeared to be mediated by intracellular IGFBP-5, independent of signalling through a cell-surface receptor, since the purification of secreted IGFBP-5 from the transfected cells followed by its addition to the culture medium of untransfected cells had no effect on cell growth (Butt et al., 2003). This may indicate that intracellular expression of IGFBP-5 interacts with different, cell surface-independent signalling pathways (Butt et al., 2003) as has been suggested by recent studies with IGFBP-3 (Butt et al., 2002). In support of this, the inhibitory and pro-apoptotic effects of IGFBP-5 on breast cancer cell growth appear to be independent of IGF signalling, as neither MDA-MB-231 nor Hs578T cells are responsive to the mitogenic and anti-apoptotic effects of IGFs (Oh et al., 1993b).

I.1.5.4 Intracelluar effects and nuclear localisation of IGFBP-5

IGFBPs are classically considered to be secreted proteins whose major functions are extracellular. However, there is now evidence that two members of this family, IGFBP-5 and -3, may also have intracellular functions, because they are transported to the cell nucleus (Firth and Baxter, 2002). It has been speculated that IGFBP-5 might also act in an intracrine fashion, perhaps serving as a cytosol-to-nuclear shuttle for its ligand, although as to why an IGF would be transported to the nucleus, when they have no known nuclear functions, remains unresolved. Nevertheless, within the multiple basic residues in the C-Terminal 201 to 218 region of IGFBP-5 there is a sequence that is similar to the bipartite NLS found in viral and mammalian transcription factors (Radulescu, 1995). Indeed, when added to cultured human tumour and breast cancer cells, exogenous IGFBP-5 was shown to be capable of cellular and nuclear entry (Amaar et al., 2002;

Schedlich et al., 1998). It has also been shown that IGFBP-5 is localized in the nuclei of vascular smooth muscle cells (VSMC) and suggested that it possesses transcription-regulatory activity that is IGF independent (Xu et al., 2004). IGFBP-3 regulates apoptosis in an IGF-independent fashion and has been shown to localixe to nuclei by interacting with nuclear receptor retinoid X receptor-alpha (RXR-alpha) within the nucleus. IGFBP-3-induced apoptosis was ablolished in RXR-alpha-konckout cells. Thus, RXR-alpha-IGFBP-3 interaction leads to modulation of the transcriptional activity of RXR-alpha and is essential for mediating the effects of IGFBP-3 on apoptosis (Liu et al., 2000).

I.1.6 IGFBP-5 actions on the Mammary Gland

The development of the mammary gland proceeds in distinct phases. In the newborn animal, there is a rudimentary system of small ducts present, which grows slowly until the onset of puberty when pronounced ductal growth occurs. Development of the ducts continues in the virgin animal leading to the formation of a ductal tree, which is made up of mammary epithelial cells sitting within adipose tissue. Extensive ductal branching and alveolar growth occurs during pregnancy and is largely completed at parturition. Terminal differentiation of the alveolar epithelium is completed at the end of gestation with the onset of milk secretion at parturition. After weaning the majority of the alveolar epithelium undergoes apoptosis, which is called involution, and the gland is remodelled back to a ductal epithelium resembling the virgin gland. The different developmental stages in the mammary gland are shown in Figure I.7.

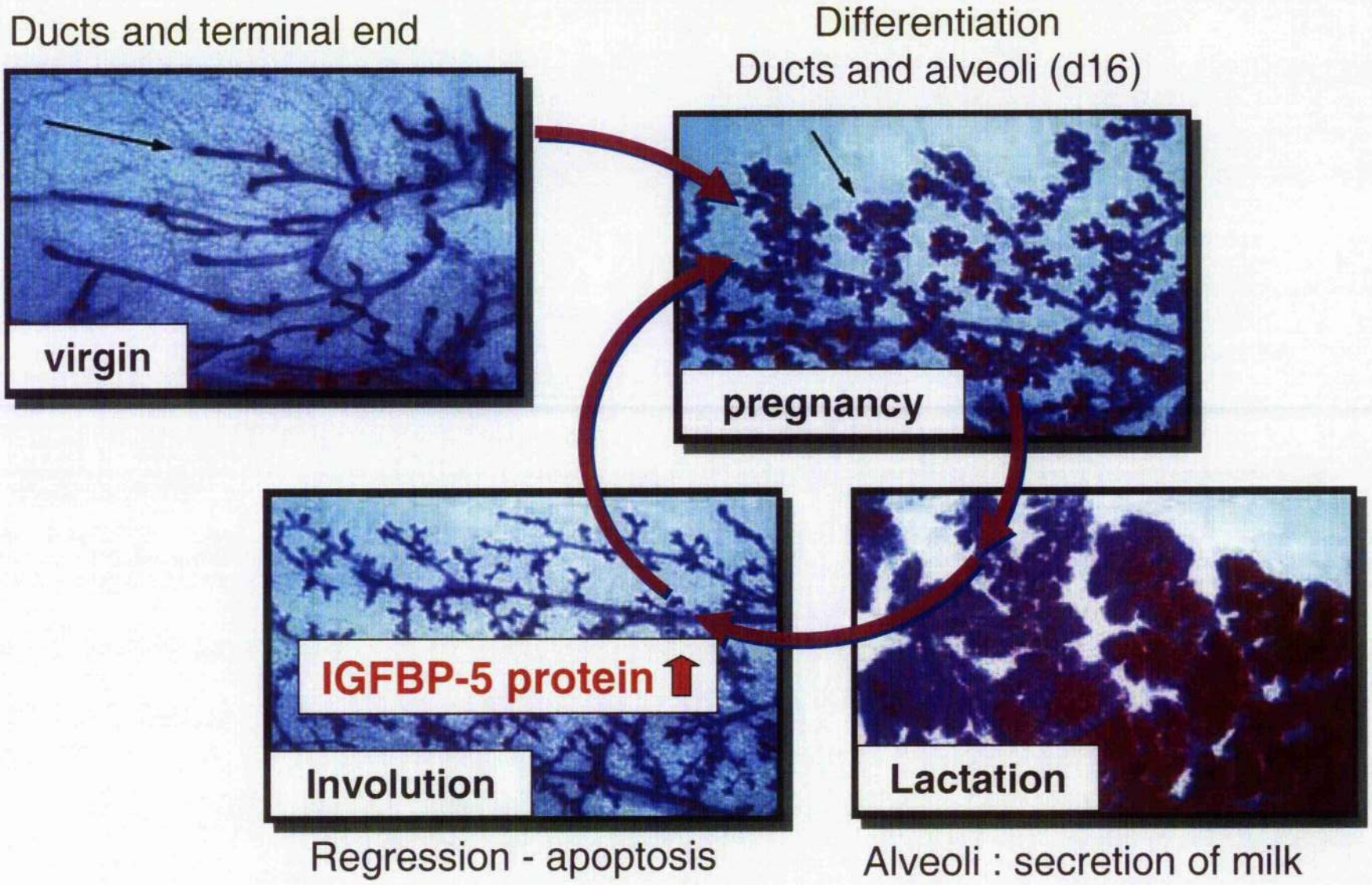


Figure I.7 Mammary gland developmental stages. The pictures show mouse mammary gland sections stained for epithelial cells.

IGF-I has been shown to suppresses apoptosis of primary mammary epithelial cells *in vitro* (Farrelly et al., 1999) and *in vivo* IGF-I and -II have been shown to inhibit mammary cell death in transgenic mouse models (Hadsell et al., 1996; Moorehead et al., 2001; Neuenschwander et al., 1996). In these mice there was a delayed involution in their mammary glands as a result of reduced levels of apoptosis. Regulating the availability of IGFs to mammary epithelial cells may therefore represent a physiological mechanism for initiating apoptosis during the process of involution (Marshman and Streuli, 2002).

It has previously been observed that an increase in IGFBP-5 protein expression in rat milk after 48 hours of involution was associated with apoptosis of mammary epithelial cells (Tonner et al., 1997). More recently, our group has shown that IGFBP-5 mRNA levels are also significantly increased during involution in the mouse mammary gland (Boutinaud et al., 2004). Therefore, it has been proposed 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.

A role for IGFBP-5 as an inhibitor of IGF-I-mediated cell survival was supported by the demonstration that the delay in involution that was observed in signal transducers and activators of transcription (STAT)-3 knock-out mice was also associated with a reduction in IGFBP-5 expression (Chapman et al., 1999), whereas in interferon regulatory factor-1 (IRF-1) knockout mice, both involution and IGFBP-5 expression were accelerated (Chapman et al., 2000). Furthermore, over-expressing the tumour suppressor PTEN (phosphate and tensin homologue) specifically in the mammary glands of transgenic mice resulted in impaired mammary gland development and microarray analysis of mammary glands from these animals revealed a 26-fold increase in the expression of IGFBP-5 (Dupont et al., 2002). This reveals that IGFBP-5 is a downstream target for PTEN, which is the second most commonly mutated tumour suppressor gene in breast cancer tumours after p53 (Li et al., 2002), and suggests that IGFBP-5 itself may have possible tumour suppression potential.

A causal relationship between IGFBP-5 synthesis and cell death in the mammary gland has now been demonstrated in three studies, where IGFBP-5 has been administered exogenously *in vitro* (Marshman et al., 2003) and *in vivo* (Allan et al., 2002) and in a transgenic mouse model involving the over-expression of IGFBP-5 using a mammary-specific promoter (Tonner et al., 2002). As discussed above, recent studies using human breast cancer cells have also revealed that IGFBP-5 can inhibit their growth both *in vivo* and *in vitro* (Butt et al., 2003).

The addition of exogenous IGFBP-5 or IGFBP-3 to mammary epithelial cells has been shown to inhibit IGF-I-mediated survival, resulting in 3-fold greater rates of apoptosis (Marshman et al., 2003). Also, subcutaneous injection of recombinant IGFBP-5 into mice during late pregnancy resulted in impaired mammary development as evidenced by reduced invasion of the mammary fat pad (Allan et al., 2002). However, the most convincing evidence comes from our group's studies in transgenic mice expressing IGFBP-5 in the mammary gland, using a mammary-specific promoter, β-lactoglobulin (BLG) (Tonner et al., 2002). The DNA content in the mammary glands of the transgenic mice relative to the wild-type controls was decreased as early as day 10 of pregnancy and mammary cell number and milk synthesis were both decreased by approximately 50% during the first 10 days of lactation (Tonner et al., 2002). At parturition concentrations of both the pro-apoptotic molecule caspase-3 and the ECM-remodelling associated molecule plasmin were both increased, whereas the concentrations of two pro-survival molecules, Bcl-2 and Bcl-xl were significantly reduced. These findings strongly support a pro-apoptotic effect of IGFBP-5 in the mammary gland *in vivo*.

Although there is a growing literature supporting the concept that IGFBP-5 is produced at sites of apoptosis, a number of studies have also identified an enhancing effect of IGFBP-5 on IGF-I action *in vitro* (Jones et al., 1993c). Augmentation of IGF action by IGFBPs has been proposed to involve proteolysis and/or reduction in the affinity of the IGFBP for IGF-1, with possible interaction with components of the ECM implicated in this event (Clemmons, 1998; Nam et al., 2000). Typically, augmentation of IGF actions takes place when IGFBP and IGF are approximately equimolar, but, when IGFBP concentrations are in excess, IGF actions are inhibited (Ewton et al., 1998). During involution of the mammary gland, IGFBP-5 has been shown to retain its high

affinity for IGF-I and concentrations of this binding protein in milk were in excess of 50 mg/l, which is several orders of magnitude greater than that of IGF-I (Tonner et al., 1997). These findings strongly suggested that the increased IGFBP-5 secretion during mammary involution was acting to inhibit IGF actions and thereby induce cell death. One possible model for how IGFBP-5 may inhibit IGF action, thereby promoting apoptosis, is shown in Figure I. 8. 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-IR on the cell surface. In turn, this would inhibit the cell survival signalling action of the IGFs and allow apoptosis to proceed.

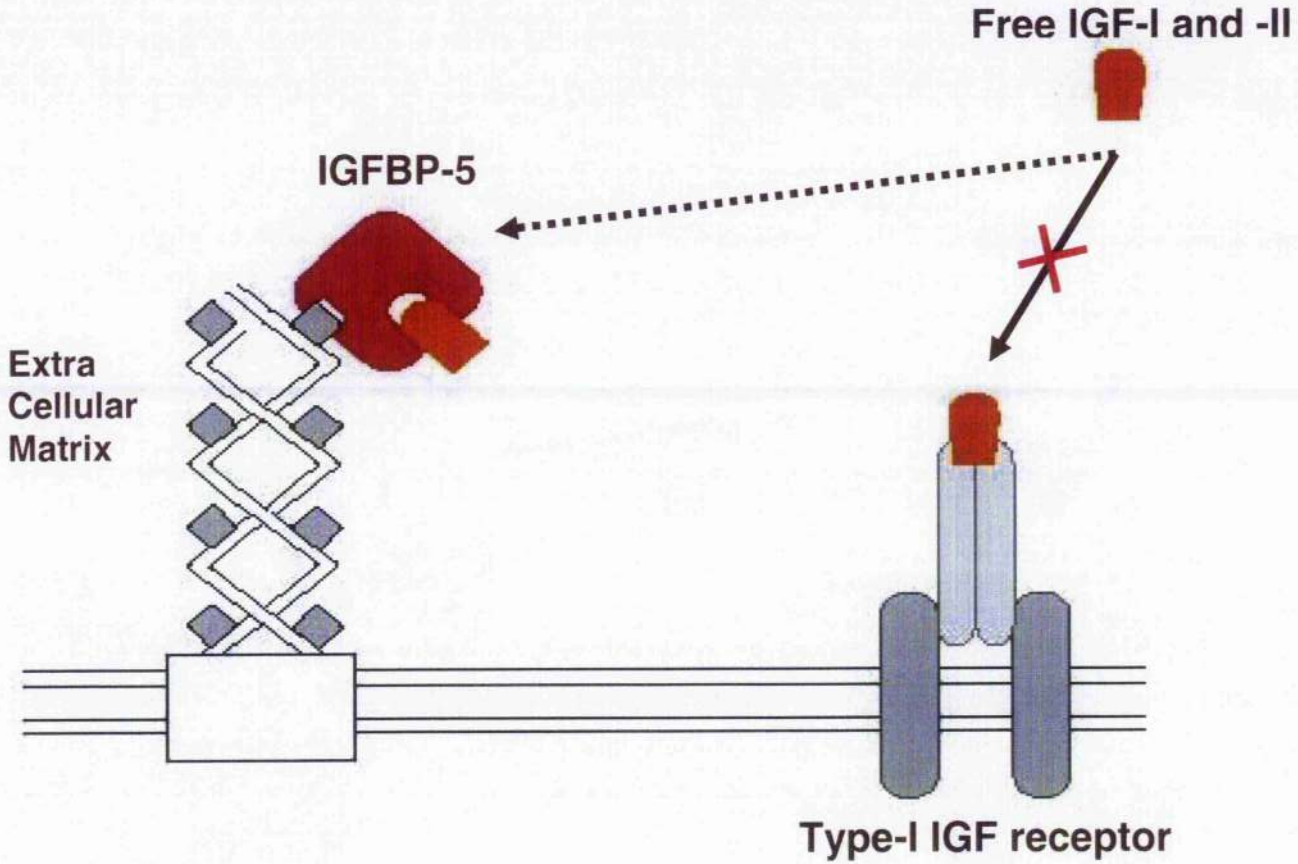


Figure I. 8 Pro-apoptotic model for IGFBP-5. IGFBP-5 promotes apoptosis by sequestering the IGFs to the extracellular matrix (ECM) and away from type-I IGF receptor (IGF-IR).

Finally, the plasminogen activation system is also involved in tissue remodelling and apoptosis, and we postulate that this could provide a further route for IGFBP-5 action. IGFBP-5 has previously been shown by others to bind to 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., 2000b). Dimeric αS_2-casein has also been shown to bind to both plasminogen and tissue type plasminogen activator (tPA), and this binding results in the enhanced activity of tPA, thereby enhancing the conversion of plasminogen to plasmin (Heegaard et al., 1997b).

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). We have postulated that this binding may inhibit the action of PAI-1, which would, in turn influence the activation of plasminogen and the consequent breakdown of the ECM that takes place during tissue remodelling (Flint et al., 2000). As PAI-I binds to tPA and inhibits its actions, it is conceivable that IGFBP-5 plays a dual role in tissue remodelling/apoptosis of the mammary gland by, A. binding to IGF-I thereby inhibiting its function through sequestration and consequently promoting cell death, and, B. binding PAI-1, inhibiting its function and thereby promoting tPA activation and consequent ECM remodelling as a result of the generation of plasmin (Figure I. 9). In support of this, both prolactin and GH inhibit tPA activity and plasminogen activation in the involuting mammary gland (Tonner et al., 2000a). In conclusion, IGFBP-5 may play a central role in co-ordinating the cell death and ECM remodelling that takes place during mammary gland involution by regulating both IGF and PAI-1 actions. Of course, it remains to be ascertained whether IGFBP-5 can bind to IGF-I and PAI-I simultaneously, although overlap of their important contact sites in the IGFBP-5 sequence may suggest that this is not straightforward.

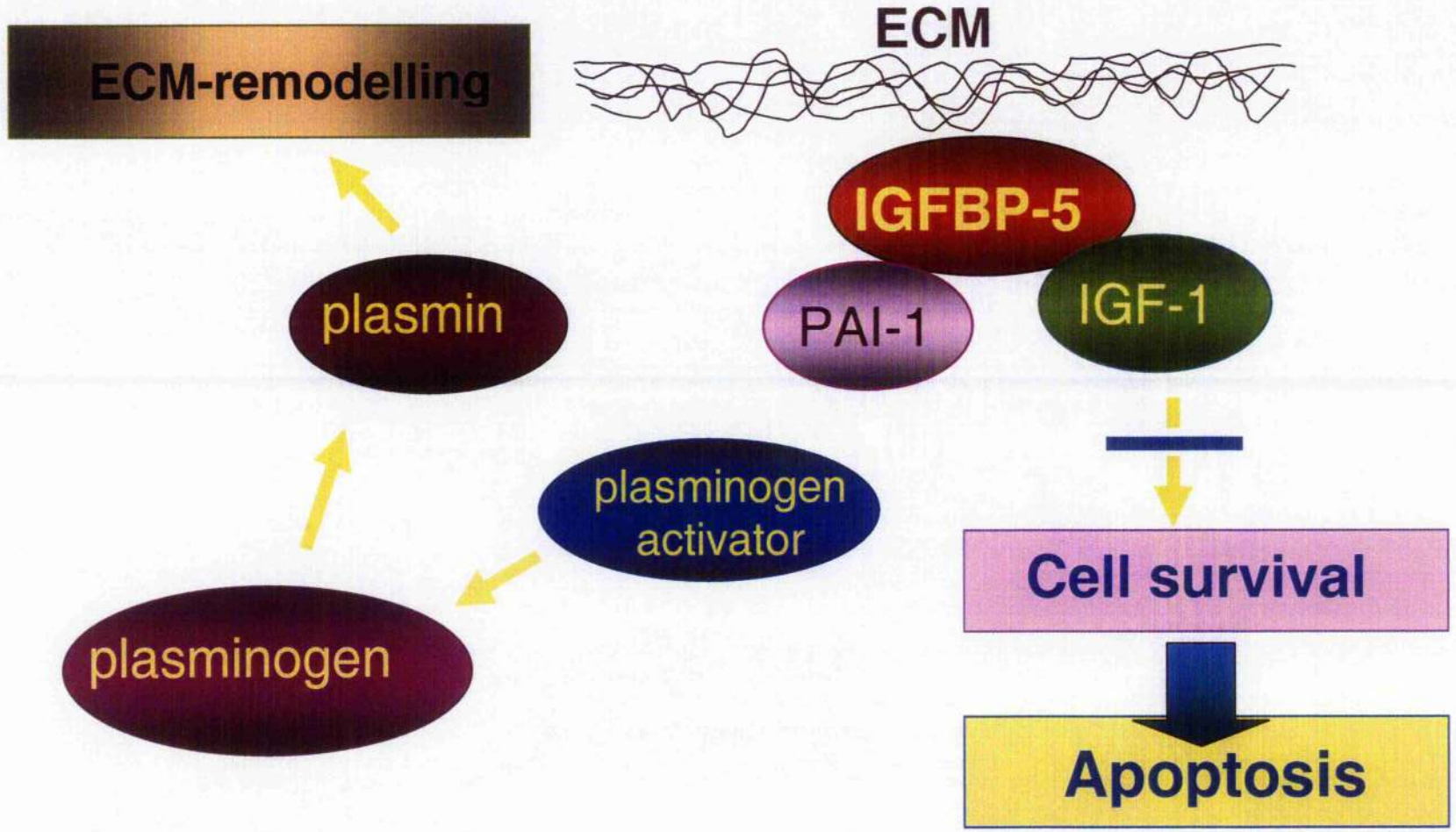


Figure I. 9 Model to describe interactions of IGFBP-5 with IGF and the plasminogen system as a possible mechnism for co-ordinating apoptosis and extracellular matrix degradation.

I.2 Regulation of IGFBP-5

I.2.1 Genomic evolution

Although the six IGFBPs have distinct amino acid sequences, they do share regions of strong homology and a similar genomic organisation (Allander et al., 1994; Kou et al., 1994a; Kou et al., 1994b; Upton et al., 1993; Zhu et al., 1993). The evolutionary conservation of IGFBPs supports their fundamental importance in regulatory processes. The IGFBP genes are physically associated on chromosomes with the homeobox (HOX) genes, which are widely expressed in multicellular organisms and encode transcriptional factors that are crucial for early development (Figure I.10). 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; Pendleton et al., 1993; Schughart et al., 1989). The human HOX gene family consists of four gene clusters, HOX A-D, localized to human chromosomes 2, 7, 12, and 17 respectively (Acampora et al., 1989; Cannizzaro et al., 1987; Scott, 1992). IGFBP-1 and -3 are localised on the same chromosome as the HOXA cluster, the IGFBP-2 and -5 genes map to the same chromosomal region as the HOXD cluster while the IGFBP-4 gene is found in the vicinity of the HOXB genes. Finally, IGFBP-6 and HOXC genes are found on the same chromosome (Figure I. 10).

Furthermore, in both humans and mice pairing is observed between IGFBP-2 and -5, and between IGFBP-1 and -3 with respect to their chromosomal locations, which could suggest co-regulation of the IGFBPs within each pair (Allander et al., 1994; Kou et al., 1994b). 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 as a result. 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 ancestral 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., 1995), and this might reflect two different evolutionary pathways after the divergence of humans and mice (Allander et al., 1995).

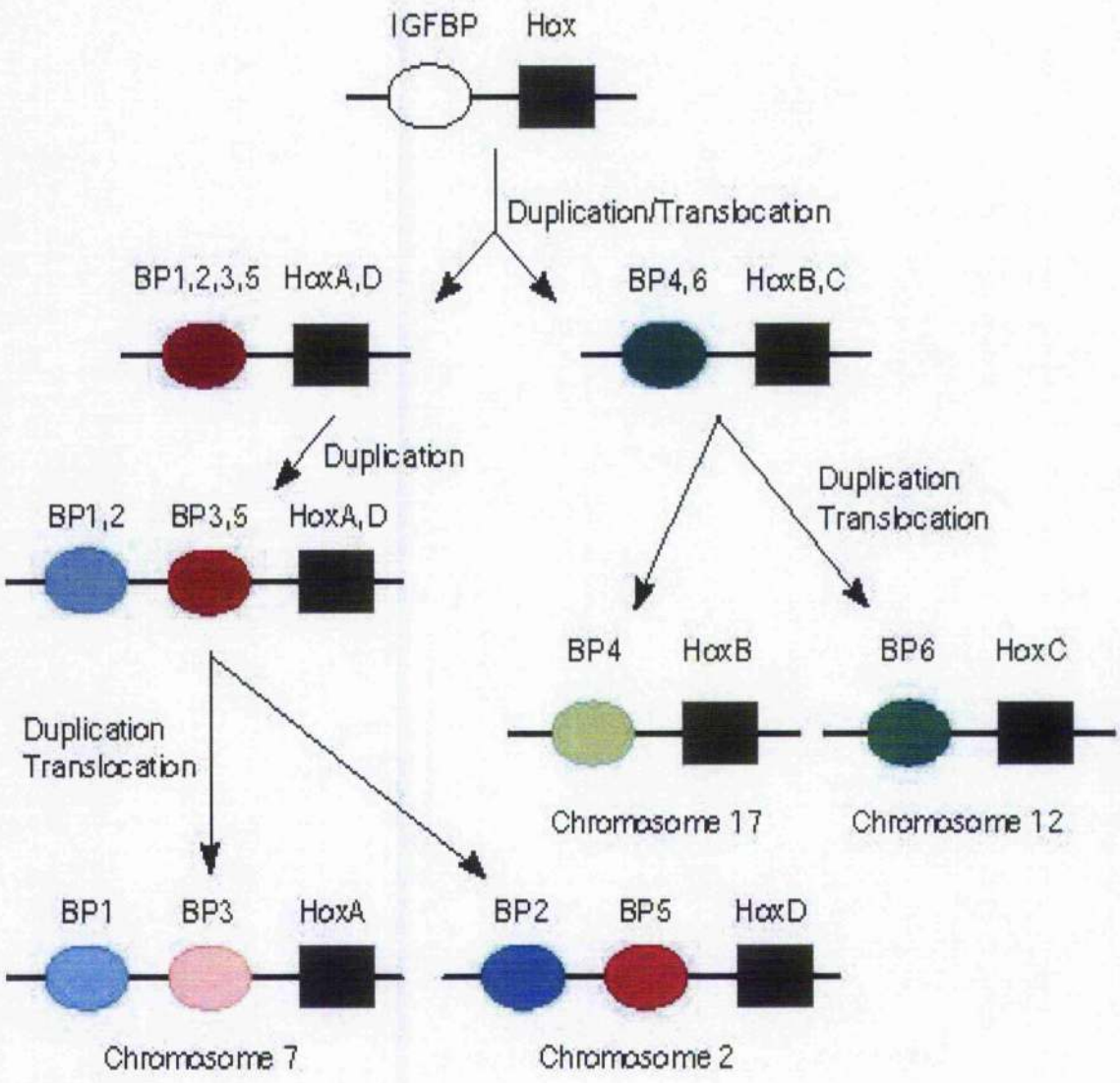


Figure I. 10 Schematic diagram of IGFBP/Hox gene evolution.

I.2.2 Genomic structure of the IGFBP-5 gene

The IGFBP-5 gene has a conserved structure of four exons separated by three introns in human, mouse and rat. To date, cDNA clones have been isolated and sequenced from human, mouse, rat and chicken (Allander et al., 1997; Kiefer et al., 1991; Shimasaki and Ling, 1991; Zhu et al., 1993). The first intron has a length of several kilobases, encompassing more than 50 % of the gene. The mouse IGFBP-5 gene has been cloned and the genomic structure and sequence of its four exons established. It spans a length of 17 kb on mouse chromosome 1 (Kou et al., 1994a). In humans, the IGFBP-5 gene spans 33 kb and is localised to chromosome 2 (Allander et al., 1994). The IGFBP-5 gene has been shown to be located on the same chromosome as IGFBP-2 in humans and mice, with the two genes orientated in a tail-to-tail fashion so that they have the opposite transcriptional direction. The distance between both genes comprises only 20 kb in humans (Allander et al., 1994) and 5 kb in mice (Kou et al., 1994a). Similarly, the IGFBP-1 and -3 genes are tightly linked and are positioned in a tail-to tail orientation on chromosome 7 in humans (Ehrenborg *et al*, 1992). The IGFBP-4 and -6 genes are located on separate chromosomes. The genomic distribution and the close relationship between certain IGFBPs suggest that these proteins have developed after duplication of an ancestral IGFBP. The resulting gene pair might then have been dispersed to different chromosomal locations (Allander et al., 1994). Since IGFBP-5 is the most conserved IGFBP between different species it appears likely that it represents the binding protein which is most similar to the ancestral proto-IGFBP.

At present, the cDNAs encoding the six rat and human IGFBPs have been isolated and characterized revealing that the six IGFBPs are clearly distinct, but share regions with strong homology (Drop et al., 1992). IGFBP gene expression is tissue specific and developmentally regulated (Rechler and Brown, 1992).

I.2.3 The IGFBP-5 promoter

The IGFBP-5 promoter has a simple structure, typical for regulated eukaryotic genes (Zhu et al., 1993). Conserved TATA and CAAT consensus sequences are present upstream of the

transcription start site in human (Allander et al., 1994), mouse (Kou et al., 1995), and rat (Zhu et al., 1993) promoters. It has been shown that there is a single transcriptional start site located 772 nucleotides 5' (or downstream) of the ATG translational start codon (Zhu et al., 1993). In addition to the TATA and CAAT boxes, there are other multiple putative cis-regulatory elements present in the promoter region, including sites for activation protein-1 (AP-1), AP-2 and a binding site for progesterone receptor (Zhu et al., 1993).

Earlier work demonstrated the orientation-dependency of DNA fragments containing different regions of upstream sequence, between nucleotide positions –31 to –4,100 relative to the transcriptional start site, of the mouse IGFBP-5 gene in directing expression of the heterologous reporter gene luciferase after transient transfection in Hep G2 cells (Kou et al., 1995). These transient transfection experiments showed that 4100 bp of mouse IGFBP-5 5'-flanking sequences and the first 120 nucelotides of exon 1 directed 25 times more luciferase expression than a promoter-less plasmid in transfected Hep G2 cells. Although a 1,004 bp nucleotide fragment was the most active in these transient transfection assays, a promoter segment containing only 156 bp of 5'-flanking DNA mediated > 60 % of the promoter's activity, and a segment comprising only the TATA box and the adjacent 5' untranslated region of exon 1 still conferred some promoter function (Kou et al., 1995). Furthermore, a 37 bp region from position -70 to -34 within the highly active 156 bp nucleotide fragment was identified as a proximal promoter that is required for full transcriptional activity (Kou et al., 1995) and this sequence is almost completely conserved in the rat and human genes (Allander et al., 1994; Zhu et al., 1993). Furthermore, this region was shown to contain a binding site for a, as yet unknown, transcription factor as evidenced by the footprint detected by DNase I protection assays, and small internal deletions within this 37-bp sequence led to a significant reduction in the activity of the intact promoter.

Further transient transfection experiments from the same group demonstrated that 1004-bp of IGFBP-5 5'-flanking sequence linked to the luciferase reporter gene resulted in levels of luciferase activity in C21 myoblast cells that were >100-fold higher than that obtained with a promoter-less luciferase plasmid control (Rotwein et al., 1995). Furthermore, a fragment containing the same 156 bp of 5'-flanking DNA described above retained >70% of maximal

activity, which was consistent with the studies in Hep G2 cells (Kou et al., 1995). The authors also showed that this 156 bp sequence mediated at least part of the differentiation-dependent rise in IGFBP-5 gene transcription in C21 myoblasts, since constructs containing this sequence produced a 2.2- to 2.8-fold increase in luciferase activity in differentiated cells compared with proliferating cells (Rotwein et al., 1995).

Work from a different group identified an AP-2 recognition sequence 5' of the TATA box and showed that AP-2 regulated basal and cAMP-dependent IGFBP-5 transcription (Duan and Clemmons, 1995). Subsequently, the same group demonstrated that transfection of IGF-I treated smooth muscle cells with 1278 bp of human IGFBP-5 5'-flanking sequence, containing the TATA and CAAT boxes and the putative AP-2 regulatory element, fused to a luciferase reporter gene resulted in very high levels of luciferase activity that were 345% higher than the controls (Duan et al., 1996).

It has also been shown that prostaglandin E2 (PGE2) and parathyroid hormone (PTH) stimulate the synthesis of IGFBP-5 in osteoblasts (Conover et al., 1993; McCarthy et al., 1994) through cAMP-dependent processes (Ji et al., 1999). The minimal DNA sequence required for basal and PGE2-stimulated IGFBP-5 promoter activity appeared to be within - 69 to –35 bp. This region adjoins the TATA box and contains, in addition to the AP-2 site, an E-box element and consensus recognition binding sequences for CCAAT enhancer binding protein (C/EBP) and nuclear factor-1 (NF-1) (Ji et al., 1999). Yet another group has shown that the stimulation of IGFBP-5 transcription by progesterone was mediated by a CACCC sequence in the proximal promoter (Boonyaratanakornkit et al., 1999).

In addition to the consensus binding motifs in the 5' IGFBP-5 flanking described above, more recent work has also revealed two potential Myb protein binding sites (Tanno et al., 2002). The first (M1) lies within the proximal region described above in close proximity to the TATA-box and overlapping the E-box element, whereas the second (M2) is much further downstream at position -429 to -424 from the transcription start site. Interestingly, these authors also present

evidence that Myb proteins can regulate IGFBP-5 transcription. Furthermore, others have shown that bone morphogenetic protein-7 (BMP-7) actually down-regulates the transcription of IGFBP-5 in primary cultures of fetal rat calvaria cells through a 21-bp control element that includes the rat homologue of the M1 site (Yeh and Lee, 2000). The BMP genes are critical in controlling proper development during embryogenesis, and, in particular, correct signalling between neighbouring epithelial-mesenchymal cell populations and are likely to play an analogous role in adult tissues that undergo developmental changes and display similar cellular interactions, such as the mammary gland or prostate.

Finally, there are three papers from different groups, which might offer a mechanistic explanation for the large up-regulation in IGFBP-5 expression that is observed at the onset of involution in the adult mammary gland. As described above, one group has shown that the up-regulation of IGFBP-5 expression in osteoblasts is partly mediated by C/EBP, indicating that the C/EBP binding motif in the IGFBP-5 promoter must be functional (Ji et al., 1999). Subsequently, a second group has shown by band shift and antibody interference assays that C/EBP delta is activated by signal transducer and activator of transcription (STAT)-3 in both growth-arrested mouse mammary epithelial cells and in the involuting mouse mammary gland (Hutt et al., 2000). This is very interesting, when you consider that a third group have shown that IGFBP-5 levels did not increase significantly in the involuting glands of STAT-3 conditional knock-out mice, which was also associated with a decrease in the levels of apoptosis in these glands (Chapman et al., 1999). These authors concluded that IGFBP-5 must be a target for STAT-3 regulation in the involuting mammary gland. However, there are no STAT-3 consensus binding motifs in the IGFBP-5 promoter. Therefore, it is conceivable that STAT-3 up-regulates IGFBP-5 expression indirectly at the start of involution, by first activating C/EBP, which in turn up-regulates IGFBP-5, although this mechanism is purely speculative and remains to be proven.

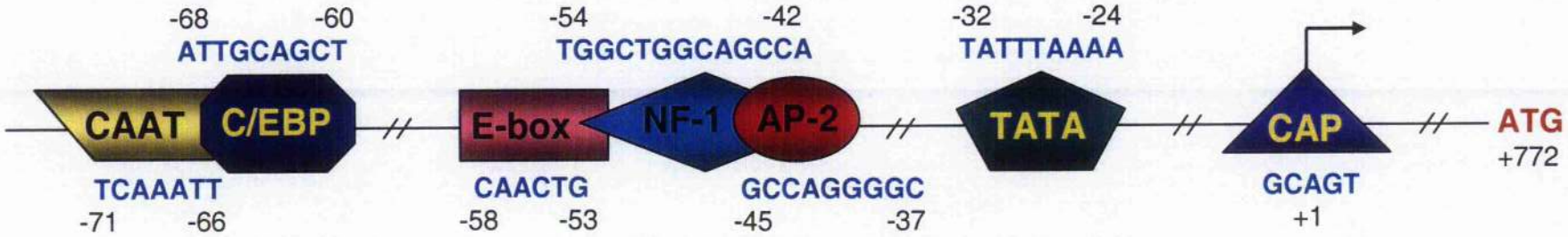


Figure I. 11 Mouse IGFBP-5 proximal promoter.

I.3 Aims of this study

This study was a continuation of our group's previous work on the structure/function of IGFBP-5, with particular reference to the role of the binding protein in cell death in the involuting mammary gland. An initial aim was to establish an *in vitro* cellular model that was relevant to the adult mammary gland and which could be used to test the regulation of IGFBP-5 expression and the function of the binding protein on cell death/survival and tissue remodelling through association with members of the plasminogen activation system.

The mouse mammary epithelial cell line, HC11, was initially chosen as these cells could be effectively induced to differentiate as measured by β-casein expression. However, it was first important to characterise the expression of the IGFBP profiles in these cells and this is presented in Chapter III in the results section. During the course of this work, we also demonstrated that there was a significant up-regulation of IGFBP-5 expression during the differentiation and death of HC11 cells, and so it was decided that these cells could provide a valuable *in vitro* model for the increase in IGFBP-5 expression that is observed upon involution of the adult mammary gland. This is presented in Chapter IV in the results section.

Finally, with respect to *in vitro* functional studies of IGFBP-5, we were also able to use HC11 cells to examine the role of IGFBP-5 in modulation of plasmin generation. This was possible because HC11 cells made sufficient concentrations of endogenous plasminogen activators to induce major morphological changes in overnight cultures and they were able to generate significant amounts of plasmin, capable of being used in a quantitative assay. This allowed us to develop a powerful *in vitro* assay to examine the effects of IGFBP-5 on the plasminogen system, and, in particular inhibition of the effects of PAI-I. This is presented in the results section of Chapter V.

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). Molecular biology reagents were from Boehringer Mannheim (East Sussex, UK), Promega (Southampton, UK) or New England Biolabs (Hertfordshire, 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 maintenance and experimental work were conducted in a MSE mistral 2000 centrifuge (MSE Loughborough, Leicestershire, UK).

General materials for cell culture. The mouse mammary epithelial cell line HC11 (Danielson et al., 1984) was a kind gift from Dr Bruce Whitelaw, Roslin Institute, Edinburgh. Sheep anti-human/rat IGFBP-5 antiserum was generated "in-house" as described below. Anti-bovine IGFBP-2 (Cat no. 06-107) was from Upstate Biotech (Charlottesville, USA). IGF-II (product no. 0M001) was from GroPep Ltd (Adelaide, Australia). Bovine insulin (I-6634), dexamethasone (D-4902), ovine prolactin (L-6520) and EGF (E-4127) were from Sigma (Dorset, UK). ^{125}Na (product no.391) was from ICN (Hampshire, UK). Sheep anti-mouse β-casein antibody was generated as described below. Tissue culture plasticware was from Corning Costar (Bucks, UK). Dulbecco's modified Eagle's medium (DMEM) (phenol red free-31053-028), Penicillin and Streptomycin (15140-122), L-glutamine (25030-024) and foetal calf serum from Gibco Brl (Scotland). Na pyruvate (P-2256) and Protease Inhibitor Cocktail (P-8340) were from Sigma (Dorset, UK). Hanks Balanced Salt Solution without Mg and Ca, Gibco cat no 14170-088, 500 ml (HBSS). Trypsin-EDTA (0.5 mg/ml trypsin, 5.3 mM EDTA), Gibco cat no 15400-054, 100ml 10x concentration diluted with HBSS to 1x. 4% Paraformaldehyde (PFA). Phosphate-buffered saline (PBS), non-sterile/sterile (filter sterilised). Crystal violet. 0.2% Triton x100. Serum-free (SF) and serum media with foetal calf serum (10%) additions. 2 μM Plasminogen, 500 μl aliquots.

Plasminogen activator inhibitor-1 (PAI-1) and plasminogen were from Calbiochem, cat nos. 528208 and 528175 respectively.

Animals All animal studies were conducted under appropriate Licence from the UK Home Office and after approval by local Ethical Review Committees. The source of mammary tissues used for these studies were from BAxC57Bl/6 strains of mice, and the developmental stages were as follows: late pregnant (18 days gestation; P18), lactating (day 1 and day 10 postpartum; L1 and L10 respectively), and involution (2 and 4 days after pup removal; I2 and I4, respectively). All animals were primiparous and litters were fixed at 8 to minimise mouse-to-mouse variation in suckling response. Animals were killed by cervical dislocation. Mammary glands were surgically removed from the mice and rapidly frozen in liquid nitrogen and stored at –80° C until use. The samples were then reduced to powder by pulverising the frozen tissue in liquid nitrogen with a mortar and pestle.

II.1 General materials and methods

II.1.1 Solutions and buffers

II.1.1.1 Nucleic acid work

Tris Borate EDTA (10 X TBE)

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

electrophoresis loading DNA sample buffer (5 X Orange G)

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

Tris-EDTA (TE) buffer

10mM Tris-HCl, pH 8.0, 1mM EDTA, Autoclaved.

Sodium chloride and Sodium Citrate (20× SSC)

3M NaCl/0.3M sodium citrate

Luria-Bertaini (LB) medium

1% *(w/v)* Tryptone (Oxoid, Hampshire, UK), 0.5% *(w/v)* Yeast extract, 0.5% *(w/v)* Sodium Chloride, pH 7.5, Autoclaved. When selection was required, ampicilin was added to the LB to give a final concentration of 100 μg/ml.

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.

II.1.1.2 Protein expression and purification

Cleavage buffer

50 mM Tris (pH 7.0), 150 mM NaCl, and 1 mM EDTA

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.

II.1.1.3 Protein assay

Cell lysis buffer

50 mM Tris.HCl (pH 7.4), 150 mM NaCl, 1% Triton-X 100, 2mM EDTA, 0.33% (v/v) Protease Inhibitor Cocktail

Tris-buffered saline (TBS-T).

20 mM Tris-HCl, 137 mM NaCl, pH 7.6 with 0.1 % Tween 20

Radioimmunoassay (RIA) buffer

50 mM NaH_2PO_4 (pH 7.4) 150 mM NaCl, 2 mM EDTA, 0.025 % (w/v) sodium azide

II.1.2 Antisera

Antirat IGFBP-5 antisera was made in house by immunizing sheep with recombinant rat IGFBP-5 protein. Antiserum was kindly donated by Dr. David Flint, and was first reported in Tonner et al. (Tonner et al., 1997). This antiserum was routinely used at a dilution of 1:2000 for Western immuno blotting.

Antirat IGFBP-2 antisera raised in rabbit was purchased from TCS Biologicals (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 1:5000 and 1:10000, respectively.

II.1.3 Iodination

Mouse IGFBP-2 and IGFBP-5 and human IGF-I were iodinated to specific activity of approximately 100 μCi/μg by the iodogen-coated tube method (Fraker and Speck, 1978). Unincorporated ^{125}I was separated from protein-bound isotope by gel filtration over a 3 ml gel column of Sephadex G10. The authenticity of radiolabelled protein was confirmed by trichloroacetic acid precipitation of product, which resulted in values typically between 80 and 90 % counts precipitated.

II.1.4 Statistics

Differences in IGFBP-2 and IGFBP-5 levels following treatment of HC11 cells with various hormonal combinations (as determined by RIA) were analysed using Student's *t*-test and were considered significant at $P<0.01$.

II.2 General techniques for characterisation of HC11 cells

II.2.1 Tissue Cultures

HC11 cells were seeded in $2.4x10^4$/well and grown to confluence in 24-well culture dishes (over a period of two to three days) in 500 μl complete medium – DMEM- 10 % FCS, 100 U/ml PenStrp, 2 mM glutamine, 1 mM Na pyruvate, 5 μg/ml insulin, 10 ng/ml EGF. Insulin and EGF are required in culture medium to produce competent cultures, which are able to respond to treatment with lactogenic hormones. After two days at confluence, competent cell cultures were induced to differentiate by treatment, under serum free conditions, with DMEM containing 5 μg/ml Insulin, 5 μg/ml prolactin and 1 μM dexamethasone (500 μl per well). Incubations were continued for

typically 2-4 days at which time conditioned medium were removed for analysis of IGFBP-5 profile and cell monolayers were lysed for the analysis of β-casein synthesis. Control, undifferentiated HC11 cell cultures were obtained by culturing competent cells in DMEM in the absence of lactogenic hormones.

II.2.2 Cell lysis

Cell monolayers were lysed in lysis buffer (Section II.1.1.3). Lysates were centrifuged for 15 min at 15800 *g* in a bench-top Eppendorf centrifuge at 4°C and supernatants were removed and stored at –20 °C prior to analysis for β-casein protein. Routinely 10 µl lysate was electrophoresed under reducing conditions and blotted for β-casein as described below.

II.2.3 Protein assay

II.2.3.1 Western immunoblot

Conditioned medium was removed from cell monolayers and centrifuged briefly to remove cellular debris. Typically 10 µl of conditioned medium or cell lysate was mixed with ×4 NuPAGE LDS sample buffer (non-reducing for ligand blotting; reducing for Western blotting). Electrophoresis was performed using Pre-cast NuPAGE 10 % Bis-Tris Gel at constant 200 V for 50 min using the manufacturer's protocol. Transfer from the gel to Hybond-C extra (Amersham) membranes was performed using the Novex Pre-Cast gel system (Gibco/Invitrogen) using the manufacturer's protocol. Western blots of cell-conditioned medium with anti-IGFBP-5 were performed in Tris-buffered saline (20 mM Tris-HCl, 137 mM NaCl, pH 7.6) with 0.1 % Tween 20 (TBS-T). After blocking for 1 hour in TBS-T/3% BSA, filters were incubated with anti-IGFBP-5 antiserum at a dilution of 1:2000 for 1 hour at room temperature or overnight at 4 °C. Following washes in TBS-T (six times 15 min), blots were probed with horseradish peroxidase (HRP)-conjugated anti-sheep (1:5000) in TBS-T/5% dried skimmed milk for 1 hour at room temperature. After drying, the protein detection was performed with the Enhanced Chemi-Luminescence technique (ECL™ Amersham, Buckinghamshire, UK) by exposing blots for various lengths of

time to Hyperfilm™ ECL™ (Amersham). Typically, membranes were exposed to autorad films (Hyperfilm βmax, Amersham) in an 18×24 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 3 min followed by a quick rinse in water, moved to GBX Kodak fixer solution for 5 min and then extensively washed in tap water.

Western blots of conditioned medium for IGFBP-2 and of cell lysates for β-casein were performed in a similar manner with the inclusion of 1% fish gelatin and 1 % BSA in TBS-T buffer for blocking and antibody dilution. The presence of fish gelatin in TBS-T/1% BSA buffers considerably reduced background levels in IGFBP-2 and β-casein blots. Primary antibodies were used at a dilution of 1:1000 (or occasionally 1:2000 for Reichmann anti-β-casein antibody). HRP-conjugated anti-rabbit secondary antibody was used at a dilution of 1:5000 for β-casein blots (Reichmann) and 1:10,000 for IGFBP-2 blots. Blots using the in-house sheep anti-rat β-casein blots were developed with anti-sheep-HRP at a dilution of 1:5000.

II.2.3.2 ^{125}I-IGF-II ligand blot

^{125}I-IGF-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 (10 mM Tris, 60 mM NaCl 0.05 % (w/v) sodium azide, pH 7.6) for 30min, with 50 ml 1% (w/v) BSA Tris-saline solution for 2hour and finally with 50 ml 0.1% (v/v) Tween 20 Tris-saline solution for 10min. Approximately 1.5-2 x 10^6cpm ^{125}I-IGF-II in 1.5 ml 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 15 min in 50 ml 0.1 % (v/v) Tween 20 Tris-saline solution and three times for 15 min in 50 ml Tris-saline solution. 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.2.3.3 Radioimmunoassay (RIA)

Levels of IGFBP-2 and -5 in undifferentiated and differentiated HC11 cell-conditioned medium were determined by RIA with the same antibodies that were used for Western blotting. For IGFBP-2 RIA, approximately 25,000 cpm ^{125}I-IGFBP-2 (100 µl) in RIA buffer (II.1.1.3) and 0-45 ng/ml unlabelled mouse IGFBP-2 protein were incubated with primary antibody (1:5000) in a final volume of 300 µl anti-rabbit precipitating antiserum [RIA buffer: 16% PEG : anti-rabbit γ-globulin : normal rabbit serum (1:1:0.08:0.008 v/v/v/v)]. Following a 1 hour incubation at room temperature and centrifugation (1700 g for 30 min), supernatants were decanted and radioactivity in pellets determined by γ-counting. An identical protocol was used in RIA of IGFBP-5 with the exception that IGFBP-5 standards were present over a concentration range of 0-750 ng/ml and precipitating antiserum contained anti-sheep γ-globulin and normal sheep serum. There was no cross-reactivity of IGFBP-2 or -5 in respective RIAs. For IGFBP-5 RIA, intra- and inter-assay coefficients of variance (determined at ED_{50}) were 13.1 and 16.8% respectively; ED_{50} was 41.5 ± 4.37 ng/ml (n=4 ± S.E.). Samples of conditioned medium were assayed at appropriate dilutions to fall into the range of the standard curves and to ensure parallelism in the assay and all samples from individual experiments were analysed in a single assay.

II.2.4 Total RNA extraction

For the total RNA extraction, HC11 cells were seeded in $8x10^5$/well and grown to confluence in 12-well culture dishes (over a period of two to three days) in 1ml complete medium. After two days at confluence, competent cell cultures were induced to differentiate under serum free conditions by treatment with DMEM containing 5 µg/ml insulin, 5 µg/ml prolactin and 1µM dexamethasone (500 µl per well). Incubations were continued for 2 days at which time cell monolayers were lysed for extracting total RNA. Undifferentiated control HC11 cell cultures were obtained by culturing competent cells in DMEM in the absence of lactogenic hormones.

Total RNA was extracted from the mammary gland samples and HC11 cells using TRIZOL Reagent according to the manufacturer's protocol (Life Technologies, Paisley, UK).

Briefly, 100 mg of mammary tissue powder was homogenised in 1 ml of TRIZOL Reagent at room temperature. For HC11 cells, 1 ml of TRIZOL was added after removal of growth medium, the cells were harvested and homogenised by passing the solution through a pipette tip several times. Chloroform (0.267 ml) was added to the homogenate and after 2 minutes incubation at room temperature, the mixture was centrifuged at 12,000 *g* for 15 minutes at 4°C. The aqueous supernatant containing total RNA was separated and 0.667 ml of isopropyl alcohol was then added for 10 minutes at –20°C to precipitate the RNA. The RNA was pelleted by centrifugation (12,000 *g* for 10 minutes at 4°C), rinsed with 70% ethanol and finally dissolved in sterile distilled water (Life Technologies, Paisley, UK). The amount of total RNA extracted from either mammary gland tissue or HC11 cells was measured by absorbance at 260 nm.

II.2.5 Reverse Transcription

A First Strand cDNA Synthesis Kit (Roche Diagnostics, Lewes, UK) was used for cDNA synthesis according to the manufacturer's instructions. 2 μg of total RNA was incubated for 1 h at 42°C with 15 units of AMV Reverse transcriptase, 1.6 μg Oligo $(dT)_{15}$ primer, RNAse inhibitor at 2.5 units/μl, deoxynucleotide mix at 1 mM, 1x reverse transcription buffer (10 mM Tris HCL, pH 8.3, 50 mM KCl) and 5 mM $MgCl_2$ in a total volume of 20 μl. After the reaction was complete, the mixture was heated to 95°C for 5 min to inactivate the AMV reverse transcriptase and chilled at 4°C.

II.3 Promoter analysis

II.3.1 Comparison of transfection reagents

To find the most efficient transfection reagent and to optimise condition for transfection into the HC11 cells, the efficacy of the various transfection reagents were compared using the pGL3-Control vector plasmid (Promega). Tfx (Promega), SuperFect (Quiagen), GeneJuice (Novagen) and FuGENE 6 (Roche Applied Science) were chosen. Transfections were performed with various amounts of DNA (0.25, 0.5, 0.75, 1μg) and various ratios of transfection reagents to

DNA [Tfx; 2:1, 3:1, 4:1/SuperFect; 2:1, 3:1, 5:1/GeneJuice; 2:1, 3:1, 4:1/FuGene; 2:3, 3:1, 6:1] using the manufacturer's protocol. Transfected cells were maintained in culture for a further 48 hr in complete medium. Cells were harvested 48 hours post-transfection and assayed for luciferase with the Promega Luciferase Assay System using the manufacturer's protocol.

II.3.2 Transient transfections and luciferase assay

HC11 cells were plated into 24-well plates at $1x10^5$ cells/ml and grown to confluence over a period of two days in 500 µl complete medium – DMEM- 10% FCS, 100 U/ml PenStrp, 2 mM glutamine, 1 mM Na pyruvate, 5 µg/ml insulin, 10 ng/ml EGF. After two days at confluence, all cells were transfected in 24-well plates with 1 µg of plasmid and a 3:1 ratio of GeneJuice (Novagen) reagent/well using the manufacture's protocol for the transfection of adherent cells and also treated in the presence or absence of lactogenic hormones [DMEM containing 5 µg/ml Insulin, 5 µg/ml prolactin and 1 µM dexamethasone (500 ul per well)] under serum free conditions. Transfected wells were maintained in culture for a further 48 hours. Cell extracts were then prepared and assayed for luciferase activity with the Promega Luciferase Assay System using the manufacturer's protocol.

II.3.3 DNA dot blot analysis

The luciferase activity for each well was normalized for the relative transfection efficiency of plasmid DNA in each well, determined by dot-blotting a portion of the cell extract on the Biotrans nylon membrane (ICN) and hybridizing this to the pGL3-basic vector (Abken and Reifenrath, 1992). In brief, a 15 µl sample from a total of 100 µl of cell extract was boiled for 5 min, diluted with 20×SSC (3 M NaCL/0.3 M sodium citrate) and dot-blotted onto nylon membrane. The DNA was UV-fixed to the membrane and hybridised to the pGL3-basic vector labelled with [^{32}P]dCTP with random primers (Feinberg and Vogelstein, 1983). The filter was then washed and exposed to a Kodak phosphor screen overnight. The resulting images were then scanned and the volumes of individual dots determined as described above. The luciferase activity was then

expressed as the luciferase activity/well divided by the relative amount of plasmid DNA/well (in arbitary units).

II.3.4 Subcloning to pGL3-basic vector

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

Agarose gel electrophoresis. 1% (w/v) agarose (Sigma) was dissolved in 1xTBE buffer and 0.5µg/ml ethidium bromide was added. Gels were electrophoresed in 1X TBE buffer at 100 V for 30 min to 1 hour. 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, Germany) following the instructions provided by the manufacturer. Electrophoresed DNA was visualised on a Herolab transilluminator (Mididoc, gel documentation analysis system and EASI store software Herolab Molekulare Trenntechnik,)

Ligation. A typical ligation reaction would be performed in 15µl volume containing: 1.5µl X 10 ligation buffer (500 mM Tris-HCl (pH7.5), 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP, 250 µg/ml BSA); 100-200 ng of linearized plamid DNA, 100-200 ng of DNA insert with compatible restriction enzyme ends and 15 U T_4DNA ligase (0.5 µl 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 10 min. 100 µl of cell suspension would be placed in pre-chilled polypropylene tubes and after the addition of 50 ng of plasmid DNA, tubes would be mixed and incubated on ice for 30 min. Tubes were incubated at 42 °C for 45 seconds and immediately chilled on ice for 2 min. After the addition of 900 µl LB medium to each tube, cells were incubated

at 37 °C for 1 hour 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. 100 µl 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 18-24 hours.

Small Scale DNA preparation. 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 the manufacturer.

Large Scale DNA preparation. Maxipreps were performed using QIAGEN tips 500 (QIAGEN, west sussex, UK) following the method recommended by the manufacturer.

II.3.5 Construction of IGFBP5-Luc series in pGL3 vector

Mouse IGFBP-5 promoter – luciferase reporter constructs: IGFBP5-Luc2, 3, 4 and 5 were kindly provided by Professor Peter Rotwein (Oregon Health & Sciences University, USA). This IGFBP-5 promoter deletion series contained different length fragments of the mouse IGFBP-5 5' flanking DNA sequence ranging from –3000 to –156 relative to the transcriptional start site: Luc2 extends to the BamHI site (-3000), Luc3 to EcoRI (-1406), Luc4 to PstI (-1004) and Luc5 to StuI (-156), with all constructs containing the same 3' end point at + 120 bp in exon 1. All of the series have been blunt end cloned into the blunted HindIII site of the pGL2-basic vector (Promega) (Kou et al., 1994a).

To subclone all of the IGFBP-5 promoter deletion series into the pGL3-basic vector, IGFBP5-Luc2, 3, 4 and 5 constructs in pGL2-basic vector were initially digested with XhoI and NarI restriction enzymes as described above. The resulting XhoI and NarI fragments were then ligated with the same sites in the pGL3-basic vector (Promega) as shown in Figure II. 1. Ligation reactions were transformed into JM109 cells, and grown on ampicillin plates for 24 h at 37°C.

Single colonies were picked and grown in LB with ampicillin selection. Mini and maxi preps were prepared as described above (II.3.4).

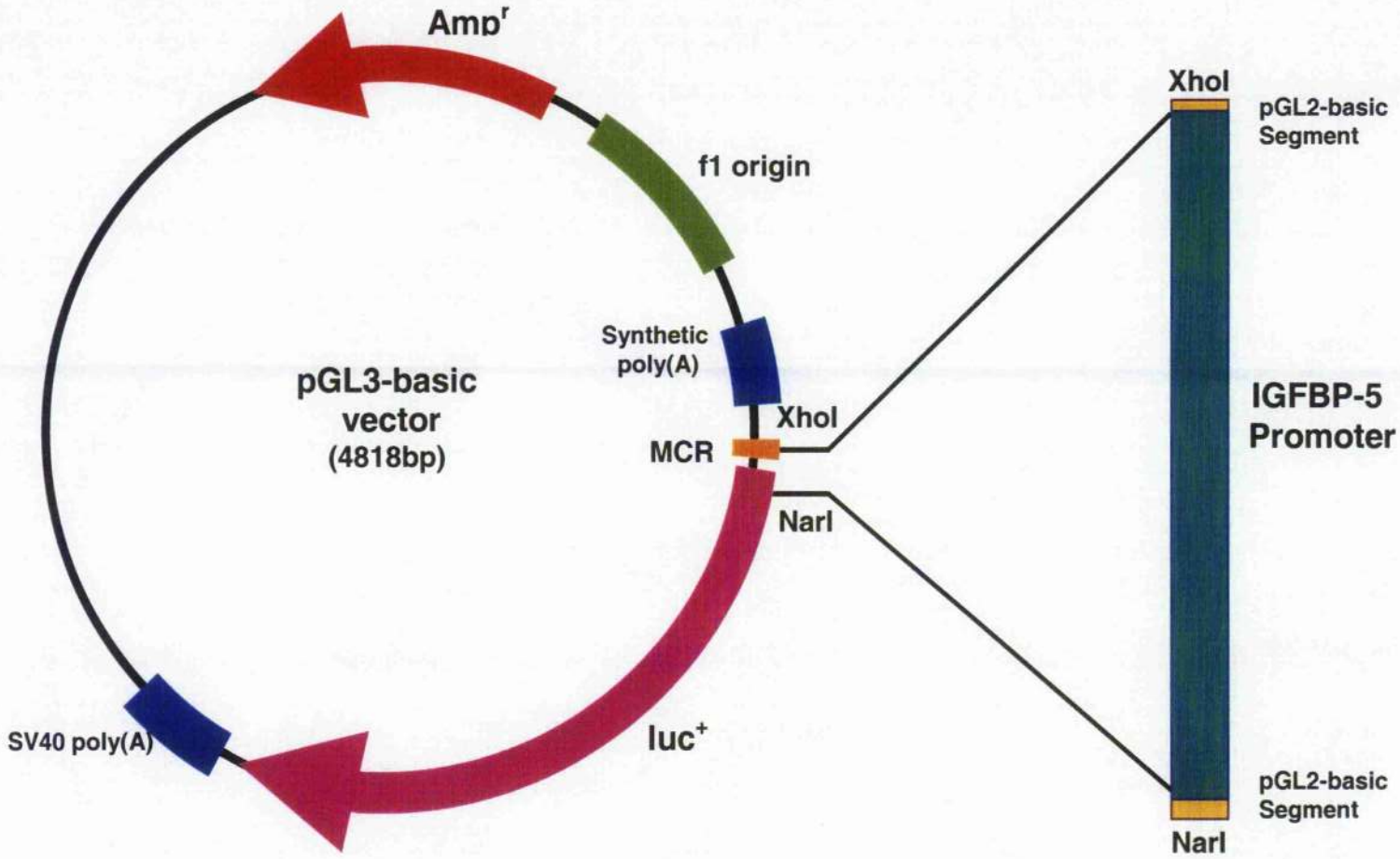


Figure II. 1 Strategy adopted to subclone the IGFBP5-Luc series into the pGL3-basic vector.

II.3.6 Site-directed mutagenesis

In order to introduce a unique XhoI restriction enzyme site necessary for creating a new IGFBP-5 promoter-luciferase deletion construct, we first had to carry out site-directed mutagenesis of the IGFBP5-Luc4 construct using the QuikChange™ system (Stratagene, La Jolla, CA, USA), following the protocol provided by the manufacturer. Site-directed mutagenesis of IGFBP5-Luc4 was carried out using the oligonucleotides: 5'-CCC TCT GCC CCT TTT AAT GCT CGA GGG GTC TAC ACA CGC GC-3' and 5'-GCG CGT GTG TAG ACC CCT CGA GCA TTA AAA GGG GCA GAG GG-3' to introduce an XhoI restriction enzyme site at position –556 and create the IGFBP5-Luc4(XhoI) construct. The structure of the new constructs and the strategy for making them is shown in Figure IV. 8. All oligos were synthesized by MWG BioTech, Milton Keynes, UK. Following site-directed mutagenesis, restriction enzyme analysis (SacI and XhoI) was performed to confirm the presence of the new XhoI site in the IGFBP5-Luc4(XhoI) construct (Figure IV.9A).

II.4 Production of recombinant mouse IGFBP-5

II.4.1 Bacterial expression of recombinant IGFBP-5

Expression of recombinant IGFBP-5 proteins (wild type and mutants) was carried out using conditions identical to those described by Allan and co-workers (Allan et al., 2002). cDNAs for mouse *wt* IGFBP-5 and various IGFBP-5 mutants minus the signal peptide-encoding sequence, were cloned into the pGEX 6P-1 vector (Amersham Pharmacia Biotech, Arlington Heights, IL) between BamHI and EcoRI in the multiple cloning site, so that the proteins would have an N-terminal glutathione *S*-transferase (GST) tag. 50 ng of this construct were then used to transform the Origami B (DE3) pLysS strain (Novagen) of *Escherichia coli*, and the cells were incubated overnight at 37 °C in 10 ml LB medium containing 12 μg/ml ampicillin and 30 μg/ml chloramphenicol. After a 40-fold dilution into fresh LB/ampicillin/chloramphenicol, the cells were re-grown to mid-log phase (E_{600nm}=~0.6), then the expression of IGFBP-5, as a glutathion-*s*-

tranferase (GST) fusion protein, was induced by addition of 1 mM isopropyl β-$_D$-thioglactoside (IPTG) and allowed to proceed at 25 °C overnight.

II.4.2 Mutant IGFBP-5

An IGFBP-5 mutant with greatly reduced affinity for IGF-I (N-term: K68N, P69Q, L70Q, L73Q, L74Q), (Imai et al., 2000; Shand et al., 2003) and two other mutants with greatly reduced affinity for heparin were used as described in the results section. The Hep- mutant (R201L, K202E, K206Q, R214A) was made as described in Song *et al.* (Song et al., 2000). The mutant (C-Term E) had all of the basic residues in the region 201-218 mutated to alanines (R201A, K202A, K206A, R207A, K208A, K211A, R214A, R216A, K217A, R218A) and mutant C-term F, based upon C-term E, but with additional mutations in the central domain of IGFBP-5 (R136A, R137A) which involved in a second putative heparin binding domain as described in Allan *et al* (Allan et al., 2006).

II.4.3 Purification of recombinant IGFBP-5

II.4.3.1 GST-affinity chromatography purification

Cells were harvested by centrifugation at 1,500 × *g* for 15 min, washed once in 50 ml PBS, and resuspended in 10 ml PBS containing 1 protease inhibitors tablet (Roche, Indianapolis, IN). The suspension was frozen and thawed once to lyse the cells, then the bacterial DNA was sheared by three 30-sec cycles of sonication (KT-40, Kontes Co., Vineland, NJ; 4-mm prove, full power) with cooling on ice. Insoluble material was removed by centrifugation at 11,000 × *g* for 30 min, then the supernatant was filtered through a 0.45-μm pore membrane and incubated overnight at 4°C with 1 ml (packed volume) washed glutathione-Sepharose (Amersham Pharmacia Biotech). The suspension was decanted into a disposable plastic column (Bio-Rad Laboratories, Inc., Hercules, CA), and unbound material allowed to flow through, then the glutathione-Sepharose was washed twice with 10 ml PBS and once with 10 ml cleavage buffer (II.1.1.2). The column was sealed, and the glutathione-Sepharose was resuspended in 2 ml cleavage buffer containing 160

units of PreScission protease (Amersham Pharmacia Biothech). After 4h at room temperature with hourly resuspension, the column was reopened, and the cleaved IGFBP-5 was recovered in the eluate. GST and PreScission protease remained bound to the glutathione-Sepharose. IGFBP-5 remaining in the column was recovered by washing with 10 ml cleavage buffer. A 400-ml bacterial culture typically yielded about 2 mg of IGFBP-5 protein.

II.4.3.2 IGF-II affinity chromatography purification

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). 500 µg of activated CM Sepharose 4B (Cat N. 17-0490- 01 Pharmacia Biotech, Uppsala Sweden) was washed in a sintered glass filter with 150 ml of 1mM HCl. Then 0.9 mg 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 100 ml of 0.05 M Tris, 0.5 M NaCl pH 8.0, then with 100 ml of 0.05 M formate, 0.5 M NaCl, pH 4.0. Excess activated CM groups were blocked with 0.1M Tris-HCl pH 8.0 and the column re-equilibrated in 50 mM Tris, 0.5 M NaCl, 0.01% sodium azide pH 7.4. The column volume was 1ml.

Protein purification was performed following the method described by Carr *et al.* (Carr et al., 1994). 800 µl of IGF-II-coupled Sepharose gel, prepared as described above, was equilibrated with 10ml equilibration buffer (0.5 M NaCl, 50 mM Tris-HCl pH 6.5). 1 ml of concentrated GST-affinity purified IGFBPs was 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 (0.5 M NaCl, 50 mM Tris-HCl pH 6.5) (the first 1ml of the flow through was retained for analysis). 6 ml of elution buffer (0.5 M acetic acid pH 3) was incubated with column gel for 10min before starting the elution. 6x 1ml fractions were collected in Eppendorf tubes already containing 300-360 µl of 2 M

Tris base necessary for immediate pH buffering to pH 7.0. Fractions were kept on ice and the column washed with 10ml equilibrium buffer (0.5 NaCl, 50 mM 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 (0.1 M Na acetate, 0.5 M NaCl, pH 4.5) were repeated twice. For storage the column was equilibrated in column storage buffer (50 mM Tris-HCl, 0.5 M NaCl, 0.01 % *(w/v)* sodium azide, pH 7.4) and kept at 4°C.

All 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 ^{125}I-IGF-II ligand blots and Western immunoblots using anti IGFBP-5 and -2 antisera. Measurement of protein concentration was by Bradford assay (see below).

II.4.3.3 Reverse Phase-High Performance Liquid Chromatography (RP-HPLC)

IGFBP-5 proteins were further purified by RP-HPLC using a polymeric column (PLRP-S: 300Å; 8 µm bead; 4.6 mm diam X 150 mm Polymer Laboratories Ltd, Church Stretton, UK) equilibrated with a mobile phase consisting of 3 part solution A [0.1% trifluoroacetic acid (TFA) in water] and 1 part solution B (0.1% TFA in acetonitrile). Aliquots (1-2 ml) of GST-affinity purified protein (2-3 mg) in cleavage buffer was injected onto the column which was run at 1ml/min throughout. After 5 min of isocratic elution, a linear gradient from 25 % solution B to 40 % solution B was applied over the next 50 min. Absorbance of column effluent was monitored at 220 nM; IGFBP-5 proteins were collected, typically between 25 and 30 min after sample injection, and fractions were assessed for protein purity by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure V. 12B). Coomassie blue staining was employed to visualise protein bands after electrophoretic separation and to estimate their relative concentration. Gels were stained in 0.3 % (w/v) Coomassie Brilliant Blue R250, 5 % (v/v) methanol, 7.5 % (v/v) glacial acetic acid by shaking gently for approximately 30 min at room temperature. To enable the detection of protein bands from the stained background, gels were destained in 30 % (v/v) methanol, 10 % (v/v) glacial acetic acid for 1 hour with shaking, followed by fresh destaining,

without shaking overnight. The gel was then dried using the EasyBreeze system (Hoefer Scientific Instruments, San Francisco, USA) according to the manufacturer's instructions. Proteins in all the fractions were also quantitated by Bradford assay. Fractions were lyophilized and stored at –70 °C. Proteins were re-dissolved in Tris.HCl/Tween 20, pH 7.4 buffer for use.

II.4.4 Protein analysis using Bradford assay

Proteins were quantified using the Bio-Rad protein assay system (Pierce, Chester, UK) which is adapted from Bradford *et al.* (Bradford, 1976). Five dilutions of a BSA standard in 0.5 % acetic acid-2 M 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 10 min at room temperature, and loaded into a TitreTeck® multi-scan and absorbance measured at 600 nm.

II.5 General techniques for functional studies of IGFBP-5

HC11 cells were routinely cultured at 37 °C in medium culture flasks (Corning) with DMEM containing 10 % *v/v* FCS (Gibco) plus 2 mM L-glutamine, 1mM sodium pyruvate, Pen/Strep (100 U/ml), epidermal growth factor (EGF) (10 ng/ml) and Insulin (5 µg/ml) for passage 12-14. At confluence cells were washed with HBSS and detached from the plastic surface by a few minutes incubation at 37 °C in the presence of 4 ml of x1Trypsin-EDTA solution (0.5 g/ℓ Trypsin and 0.2 g/ℓ EDTA, obtained by diluting x10 concentrated solution with HBSS). 15-20 ml of 10% FBS DMEM medium were added to inactivate trypsin, then cells were harvested in 50 ml centrifuge tubes and centrifuged at 1000 rpm for 5min. Supernatant was discarded and cell pellets resuspended in complete medium.

After trypsinisation, 100 µl of diluted cell suspensions were seeded into 96-well flat bottom tissue culture plates and grown to confluence for 1-2 days in DMEM or RPMI with 10 % Foetal Calf Serum (FCS) plus 2 mM L-glutamine, 1mM sodium pyruvate, Pen/Strep (100 U/ml), epidermal growth factor (EGF) (10 ng/ml) and Insulin (5 µg/ml). When cells reached confluence, the competent cell cultures were washed with serum free medium and treated with various combinations of plasminogen, plasminogen activator inhibitor-1 and *wt* IGFBP-5 or mutant proteins under serum free conditions. For the control, HC11 cells were cultured in DMEM in the absence of serum. Incubations were continued for typically 24-48 hours at which time conditioned medium were removed for analysis of plasmin activity, while cell monolayers were washed with PBS and treated with 4 % PFA for histological analysis. The concentrations of these reagents are indicated in the relevant Figures and accompanying legends. Each treatment was done in duplicate or triplicate.

II.5.1 Histological analysis of HC11 cells

After 24-48 hours incubation with various combinations of proteins, HC11 cells were washed with PBS twice to remove traces of medium. 100ul of 4% paraformaldehyde (PFA) was added to each well for 20 min to 2 hours in order to fix the cells. Wells were then washed twice with PBS. The cells were then stained with 100 µl crystal violet for 5-30 minutes and washed with double distilled water (ddH2O) until clear, before microscopic analysis and photography was carried out.

II.5.2 Assay for plasmin activity

Plasmin activity was measured by incubating conditioned media in 50 mM Tris HCl, 0.1% Tween 20, pH 7.4 with 25ul of the artificial substrate 6mM H-Val-Leu-Lys-p-nitroanilide (VLL-pNA) from Bachem UK (0554101). The absorbance at 405 nm was measured at 15 min intervals for 4 hours and plasmin activity was determined by the rate of change in absorbance.

II.5.3 Cell-free-system plasmin assay

The assay described above is dependent on the fact that HC11 cells are known to express their own plasminogen activator (PA). However, we also wanted to employ a cell-free system in which we could control the amount of PA present. Initially, we examined the effect of tPA and urokinase plasmin activator (uPA) alone on plasmin generation. First, tPA (typically 50 ng/ml) or uPA (typically 1.1 μg/ml) was added to a 96-well microtitre plate containing 10 μl of 2mM plasminogen, 10 μl of 6 mM VLL-pNA and made up to 100 μl/well with 50 mM Tris.HCl plus 0.1% Tween 20 pH 7.4. Thereafter, absorbance at 405 nm was measured at 15 min intervals for 4 hours and the generation of nitroaniline was determined by the rate of change in absorbance. Second, to test the relationship between IGFBP-5 and tPA/uPA, a range of concentrations of IGFBP-5 (500 μg/ml, 250 μg/ml, 125 μg/ml) were added to plasminogen, tPA/uPA, VLL-pNA and Tris.HCl, 0.1% Tween 20 pH 7.4 and plasmin activity was measured as described above. The control was bovine serum albumin (BSA) at the same concentrations (500 μg/ml, 250 μg/ml, 125 μg/ml) instead of IGFBP-5.

Chapter III. Characterisation of HC11 cells

III.1 Introduction

The cycle of various mammary gland developmental stages requires the coordinated action of growth factors and hormones that promote morphological development and milk protein production (Topper and Freeman, 1980). The IGF axis plays an important role in an autocrine and paracrine manner in this process (Hadsell et al., 1996; LeRoith et al., 1995; Neuenschwander et al., 1996; Ruan and Kleinberg, 1999). In order to understand the IGF axis contribution to mammary gland development and differentiation, an *in vitro* cell culture system has been developed.

The HC11 mouse mammary epithelial cell line may serve as a useful model system for studying functional characteristics of mammary cell differentiation, because primary mammary epithelial cells require either co-cultivation with mesenchymal cells or with exogenous ECM components to differentiate in the presence of lactogenic hormones and produce milk proteins. HC11 cells however are an exception, since they produce large quantities of β-casein, a marker of epithelial cell differentiation, when lactogenic hormones are added to confluent cells previously grown in the presence of EGF and insulin even in the absence of mesenchymal cells (Ball et al., 1988a; Ball et al., 1988b; Merlo et al., 1996). This cell line was clonally derived from the heterogeneous COMMA-1D line isolated from a mid-pregnant Balb/c mouse mammary gland (Danielson et al., 1984).

This study demonstrates the characterisation of the IGFBP secretion profile in relation to undifferentiated and differentiated HC11 cells and analyses the hormonal regulation of the IGFBP-2 and -5 by HC11 cells by dexamethasone (D), insulin (I), and prolactin (P). In addition, the mRNA profile of IGFBPs was ascertained using quantitative RT-PCR during various stages of murine mammary gland developments. Moreover, a study was undertaken to examine similarities and differences in IGFBP mRNA expression profiles between mammary gland and HC11 cell line.

III.2 Results

III.2.1 IGFBP secretion profile of HC11 cells

III.2.1.1 IGFBP-5 expression increases during HC11 differentiation

Initial analysis of the profile of IGFBP secretion by undifferentiated and differentiated HC11 cell cultures was performed using ligand blotting with ^{125}I-IGF-II and Western Blotting with specific antisera to IGFBP-2 and -5. Figure III.1 illustrates the profile of IGFBP secretion into the conditioned medium of undifferentiated (U) and differentiated (D) cell cultures. Ligand blot analysis using radio-labelled IGF-II (^{125}I-IGF-II) (Figure III.1A) indicated that an IGF binding species of approximately ~30 kDa was expressed in both the undifferentiated and differentiated HC11 cell cultures. This species was only weakly present in the conditioned medium from undifferentiated cells, but significantly up-regulated in the differentiated HC11 cell culture medium. The identity of this up-regulated binding protein(s) was determined by probing Western blots of the conditioned culture medium from undifferentiated and differentiated HC11 cells with specific anti-IGFBP antibodies.

In the IGF-II ligand blot, there was no higher molecular mass doublet (~40-50 kDa) typical of IGFBP-3 in HC11 cultures or a lower molecular mass IGFBP species at ~24 kDa characteristic of IGFBP-4 (Boney et al., 1994), so we didn't consider it likely that the IGFBP species could be either IGFBP-3 or -4. Therefore, we probed HC11 cell culture medium from undifferentiated and differentiated with an antiserum to IGFBP-5 or anti-IGFBP-2. Probing with IGFBP-5 antiserum revealed IGFBP-5 (~ 30kDa) to be a binding protein species that is present in the conditioned medium from undifferentiated cells and highly up-regulated in the medium from differentiated cells (Figure III. 1B upper panel). Several smaller fragments were also detected on the anti-IGFBP-5 blot, which were not detected by IGF-II ligand blot analysis. These fragments were confirmed as proteolytic fragments of native full-length IGFBP-5 by Competitive Western analysis (data not shown). IGFBP-2 was also identified as a binding protein species present in undifferentiated conditioned medium and which was down-regulated during HC11 cell differentiation (Figure III.1

bottom panel). It is important to note that no cross reactivity was seen between the anti-IGFBP-2 and anti-IGFBP-5 antiserum. We were not able to determine the presence of either IGFBP-1 or -6 in HC11 culture medium since we could not obtain appropriate antisera for these IGFBPs.

III.2.1.2 Time course of IGFBP-5 secretion

The secretion of IGFBP-5 by differentiated HC11 cells was also confirmed in a time course experiment (Figure III. 2), where IGFBP-5 accumulated in differentiated and undifferentiated HC11 cultures over a 0-6 day time course. The secretion of ß-casein, a marker of differentiation, was analysed in parallel over the same period of time. Although accumulation of ß-casein at day 6 was decreased, IGFBP-5 secretion and ß-casein accumulation demonstrated a similar pattern for up to 6 days of culture. Interestingly, there appeared to be some accumulation of IGFBP-5 by day 6 in undifferentiated HC11 cell culture as well as differentiated cell culture.

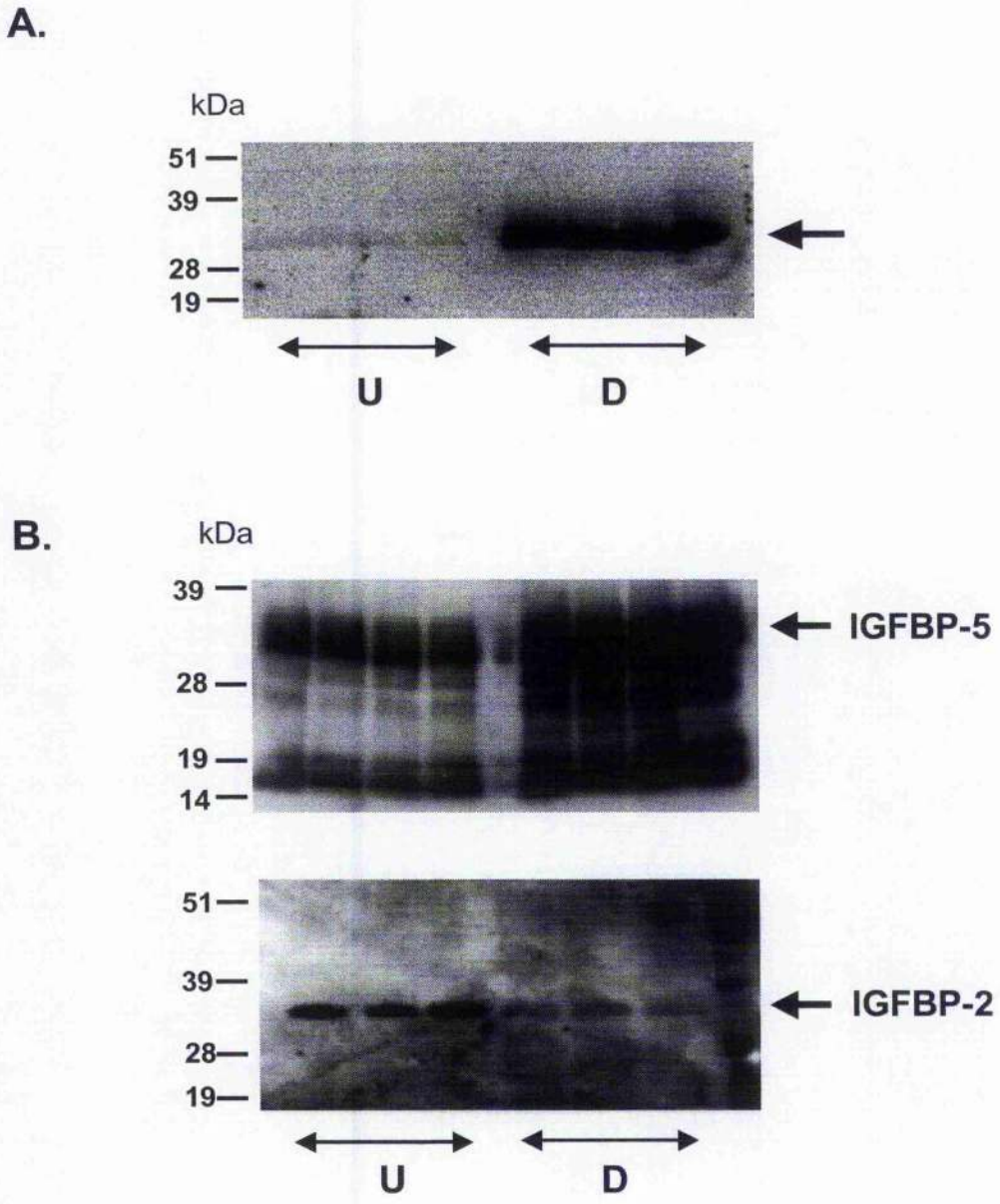


Figure III.1 IGFBP-2 and -5 are reciprocally regulated during the differentiation of HC11 cells. A. ^{125}I-IGF-II ligand blot B. Western Blot analysis identifies IGFBP-5 was upregulated in differentiated cells. Conditioned medium from undifferentiated (U) or differentiated (D) HC11 cells was blotted with an antiserum specific for IGFBP-5 (upper panel) or specific for IGFBP-2 (lower panel). IGFBP-5 secretion is clearly upregulated during HC11 cell differentiation. Conversely IGFBP-2 levels are higher in medium conditioned by undifferentiated HC11 cells and this IGFBP species is down regulated during HC11 cell differentiation.

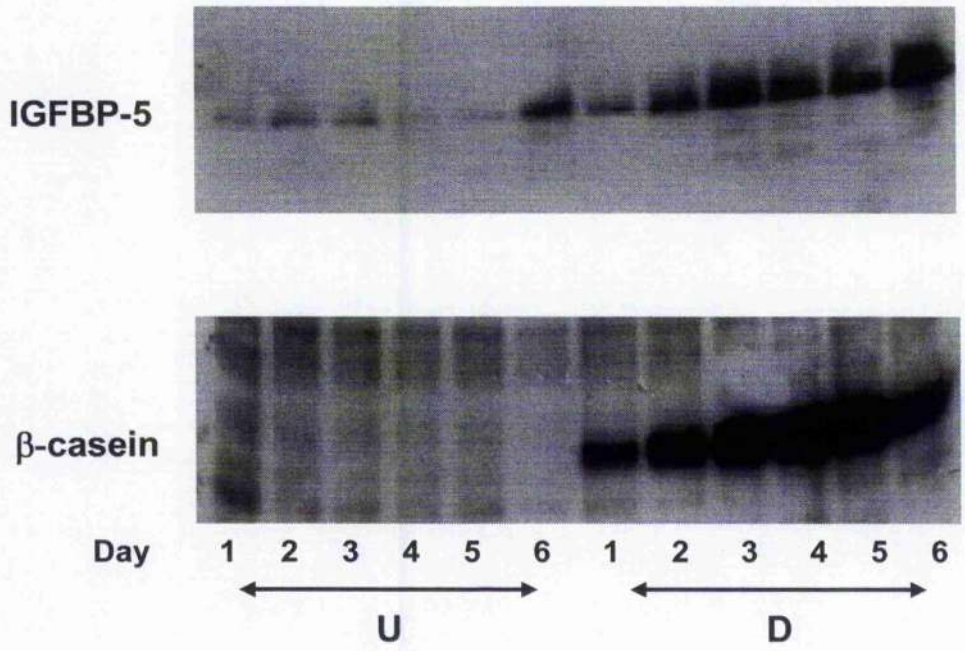
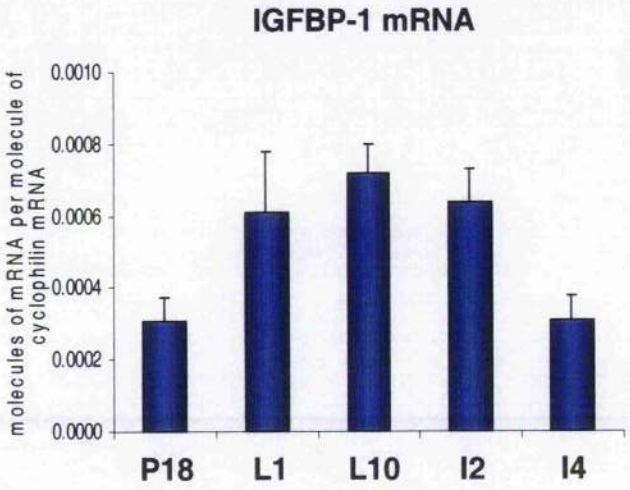


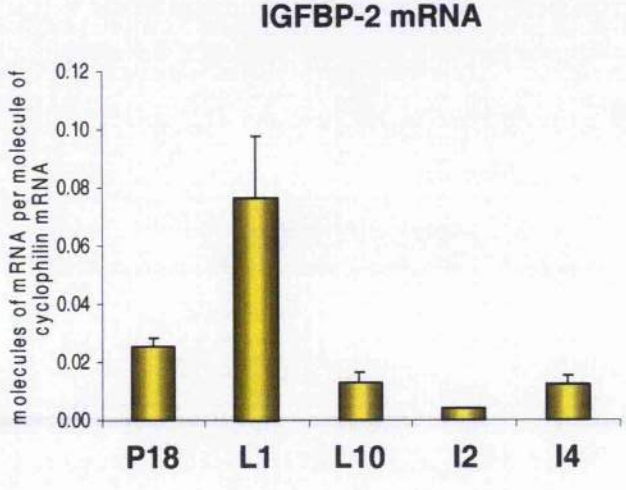
Figure III 2. Time course of IGFBP-5 secretion and β-casein synthesis by HC11 cells. Conditioned medium and HC11 cell lysates derived from undifferentiated (U) or differentiated (D). HC11 cells were analysed for IGFBP-5 and β-casein respectively at 24 hr intervals over the time period 0-6 days.

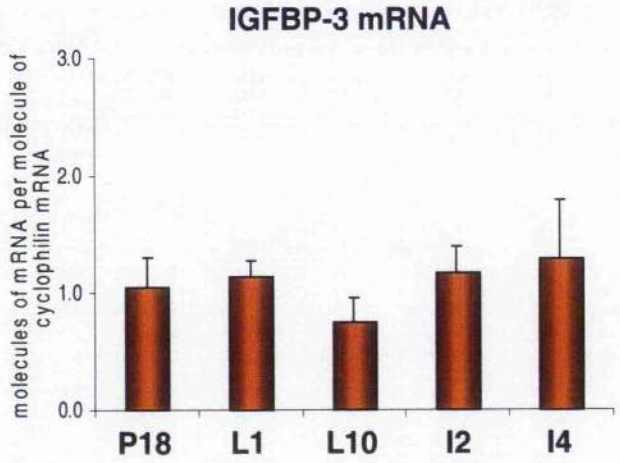
III.2.2 IGFBP mRNA expression profile

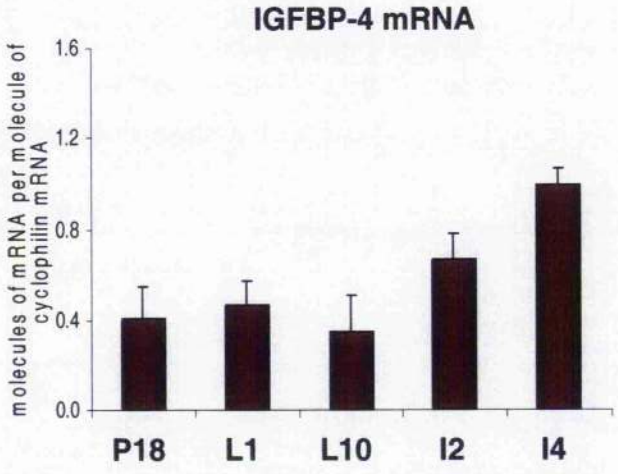
III.2.2.1 *In vivo* expression of IGFBP mRNA in the mammary gland

The mRNA profile of IGF axis components in various stages of mouse mammary gland development was explored from late pregnancy, lactation and day 4 of involution using quantitative RT-PCR. Please note that with the quantitative RT-PCR experiments reported in this chapter, although the mRNA extraction was carried out by both A. Sorrell and M. Boutinaud, data acquisition and analyses with the Roche Lightcycler was carried out exclusively by M. Boutinaud and subsequently published in (Boutinaud et al., 2004). The mRNA profile of the IGFBPs diverged according to the developmental stages of mouse mammary gland (Figure III. 3). The expression of IGFBP-1 was extremely low at all stages. Although IGFBP-2 expression was relatively low at all stages, it showed a 3-fold increase at parturition, followed by a significant 6-fold decline by lactation day 10 which persisted into involution (See details in Boutinaud et al., 2004). IGFBP-3 was expressed at high levels at all stages and its expression was not significantly different at any stage examined. IGFBP-4 expression was also relatively high and showed only an involution-specific increase. IGFBP-5 was expressed at very low levels during late pregnancy and lactation, but displayed a dramatic 54-fold increase by involution day 2, before declining again 2-fold by involution day 4. It is clear from Figure III.3 that the levels of IGFBP-5 mRNA expression during involution were considerably higher than any other IGFBP at any of the developmental stages analysed. Finally, IGFBP-6 was expressed at significant levels during pregnancy, but declined 4-fold during lactation before increasing three-fold again between involution day 2 and 4.


IGFBP-1 mRNA
molecules of mRNA per molecule of cyclophilin mRNA
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
P18
L1
L10
I2
I4


IGFBP-2 mRNA
molecules of mRNA per molecule of cyclophilin mRNA
0.12
0.10
0.08
0.06
0.04
0.02
0.00
P18
L1
L10
I2
I4


IGFBP-3 mRNA
molecules of mRNA per molecule of cyclophilin mRNA
3.0
2.0
1.0
0.0
P18
L1
L10
I2
I4


IGFBP-4 mRNA
molecules of mRNA per molecule of cyclophilin mRNA
1.6
1.2
0.8
0.4
0.0
P18
L1
L10
I2
I4

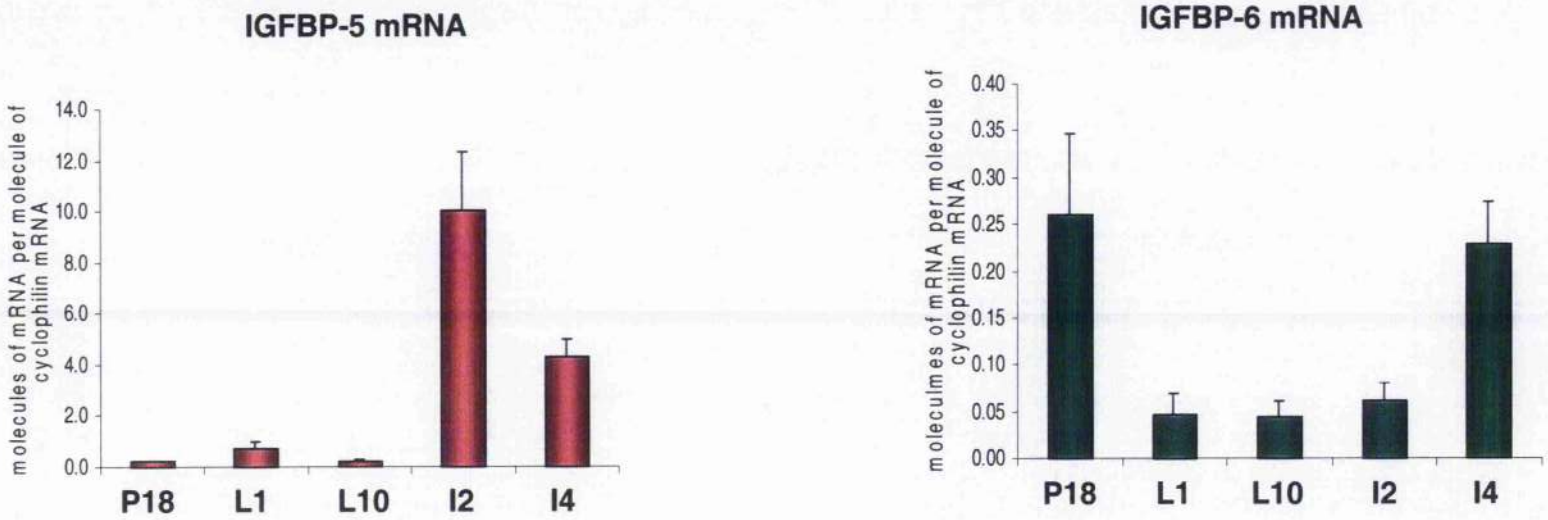


Figure III. 3 Comparison of the number of mRNA molecules per molecule of cyclophilin mRNA for all six IGFBPs in the different developmental stages of the mammary gland studied. Pregnancy day 18 (P18), Lactation day 1 and 10 (L1, L10), Involution day 2 and day, 4 (I2, I4).

III.2.2.2 *In vitro* expression of IGFBP mRNA in HC11 cells

The *in vitro* expression of IGFBPs in HC11 cells was investigated as a parallel to the *in vivo* study of IGFBP expression profiles during mammary gland developmet. Using quantitative RT-PCR, we identified *in vitro* expression of IGFBP-1, -2, -3 and -5 in HC11 cells, whereas no mRNA was detected for IGFBP-4 and -6 (Figure III. 4). IGFBP-3 and -5 were the most highly expressed binding proteins in HC11 cells. DIP treatment of HC11 cells led to a decrease in IGFBP-2 and -3 expression and an increase in the expression of IGFBP-5. There was no significant effect on the expression of IGFBP-1 by DIP treatment.

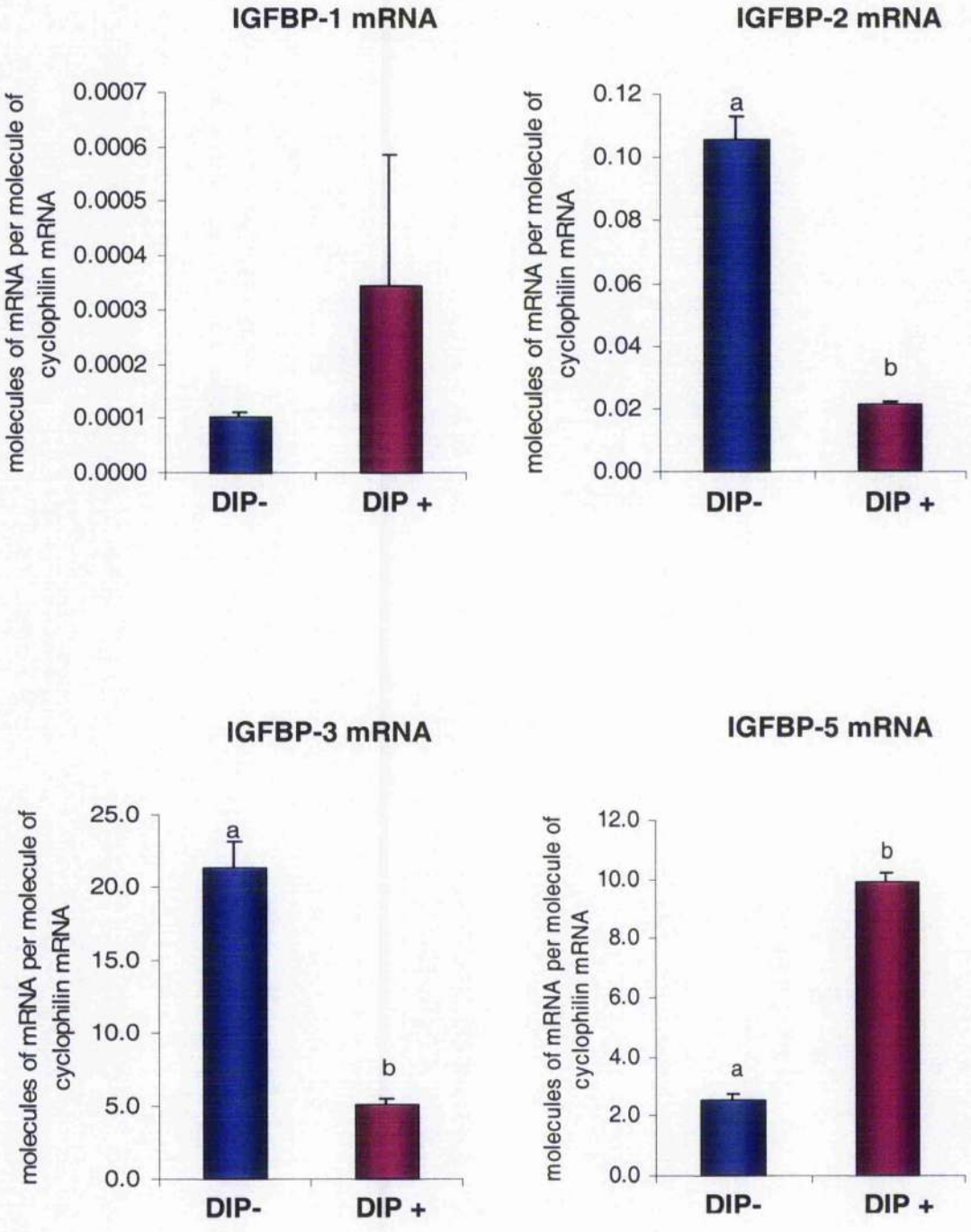


Figure III. 4 IGFBP mRNA expression profile in HC11 cells with or without DIP.

III.2.3 Hormonal control of IGFBP-2 and -5 in HC11 cells

The contribution of each components of the lactogenic hormones (D, I and P) responsible for the IGFBP secretion was assessed in order to examine whether the differentiation of HC11 cells could be dissociated from IGFBP-2 and -5 secretion. The expression levels of intracellular β-casein and secreted IGFBP-5 and -2 were scrutinized with various combinations of each lactogenic hormones (D, I and P) using western blot and radio immuno-assay (RIA).

III.2.3.1 Binary combinations of D, I, P permit IGFBP secretion to be dissociated from HC11 cell differentiation

Figure III.5 demonstrate that ß-casein synthesis in HC11 cells required the complete lactogenic complement of hormones (DIP), since treatment with binary combinations of D, I and P did not stimulate ß-casein synthesis (top panel). However, although the highest levels of IGFBP-5 were detected in HC11 culture medium with DIP, there was also a strong stimulation of IGFBP-5 expression with binary combinations of hormones (middle panel). The western blot analysis of IGFBP-2 secretion in HC11 cell conditioned medium revealed strong expression of IGFBP-2 in HC11 conditioned medium treated with the binary combination of hormones IP, but not with the other binary combinations of DI or DP. Therefore, these results imply the dissociation between the differentiation of this cell line and the observed profile of IGFBP secretion.

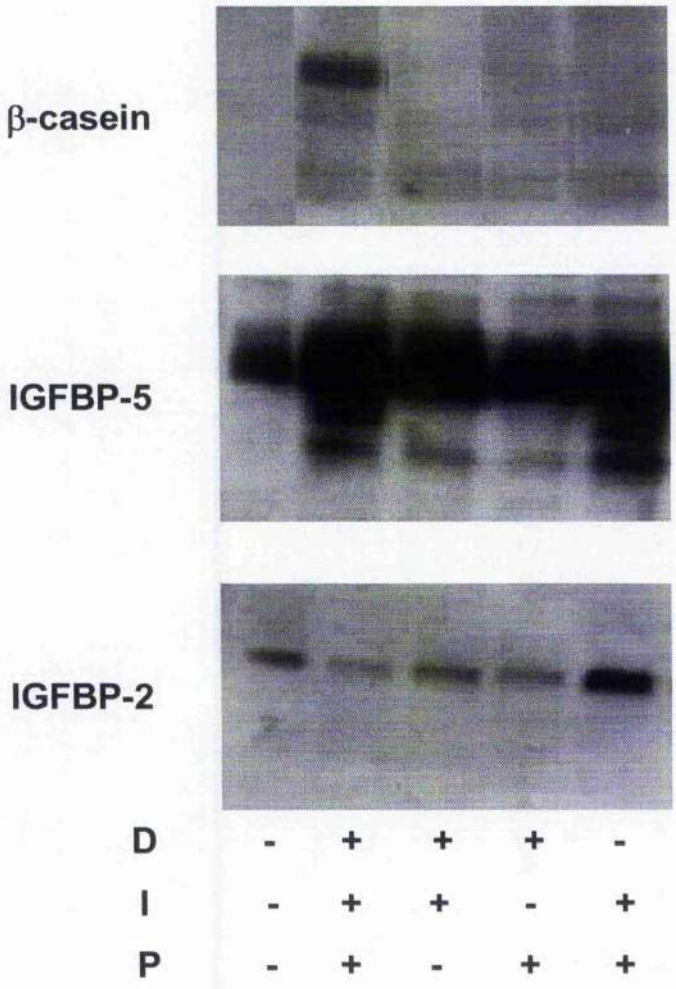


Figure III. 5 Hormonal regulation of β-casein, IGFBP-2 and -5 expression on HC11 by various combinations of D, I, P treatment. The HC11 cell lysate and conditioned medium were analysed by western blot with anti-β-casein antiserum and anti-IGFBP-2 or -5 antiserum respectively. Western blots are representative of results repeated on five occasions.

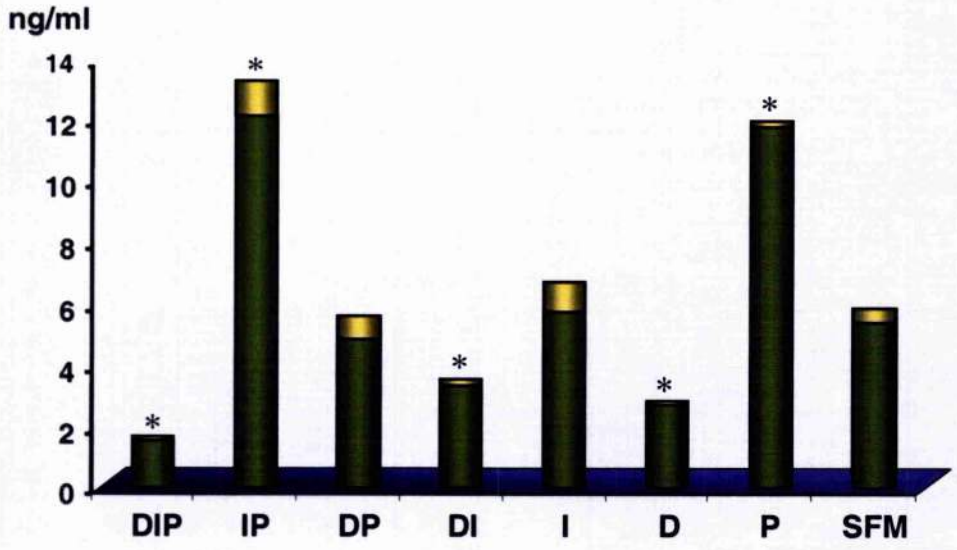
III.2.3.2 Radio-immuno assay (RIA)

IGFBP-2 and -5 secretion into HC11-conditioned medium with different combinations of lactogenic hormone mixture was quantified more precisely by radio-immuno assay using radio labelled IGFBP-2 and -5.

The IGFBP-2 RIA result indicated that insulin had no effect on the secretion of this protein. Prolactin consistently stimulated the secretion of this binding protein, whereas dexamethasone was consistently inhibitory, especially in the presence of both I and P (Figure III. 6A, DIP treatment). This indicates that the overall inhibitory effect of dexamethasone on the levels of IGFBP-2 secretion can override the stimulatory effects of prolactin. As shown in Figure III. 6B, the concentration of IGFBP-5 secreted from serum free HC11 cell culture medium was ~1 μg/ml and this increased by ~10-fold up to ~10-12 μg/ml with DIP treatment. The level of IGFBP-5 expression was stimulated when the HC11 cells were treated with binary combinations of D, I and P, which was consistent with the western blot results above. There was also a stimulatory effect of insulin or prolactin alone on IGFBP-5 levels. However, the IGFBP-5 secretion level was reduced significantly with dexamethasone treatment alone. It appeared to be that the effects of dexamethasone switched depending on whether it was used alone or in combination with P and/or I. On its own, dexamethasone inhibited IGFBP-5, whereas it augmented the effects of P and I when used in combination. This may mean that dexamethasone is necessary to prime the cells for differentiation, so that P and I are able to elicit their effects.

The most significant finding from the RIA results was the observation that the levels of IGFBP-5 secretion in HC11 conditioned medium were three orders of magnitude higher than the those for IGFBP-2 (μg/ml IGFBP-5 vs ng/ml IGFBP2). This explains why previous ligand blot analysis mainly reflected IGFBP-5, but not IGFBP-2 secretion (see Figure III.1).

A.


IGFBP-2
ng/ml
14
12
10
8
6
4
2
0
*
*
*
*
*
*
DIP
IP
DP
DI
I
D
P
SFM

B.

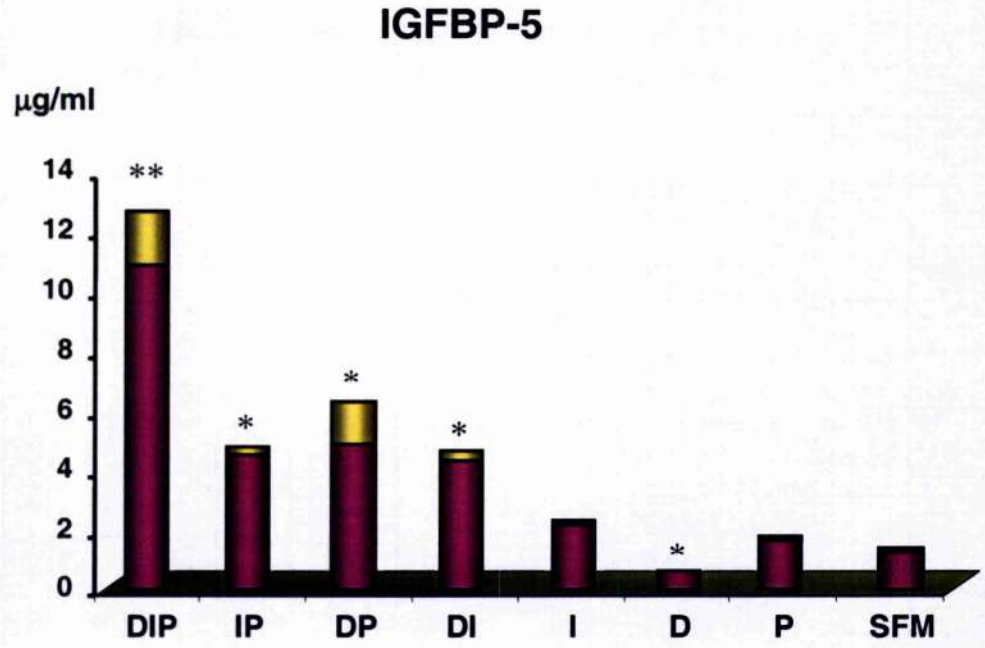

IGFBP-5
µg/ml
14
12
10
8
6
4
2
0
**
*
*
*
*
DIP
IP
DP
DI
I
D
P
SFM

Figure III. 6 Quantification of IGFBP expression levels in HC11 conditioned medium by specific RIA. A) IGFBP-2, B) IGFBP-5 RIA. Three experiments were performed with duplicate wells for each treatment and data are pooled as mean ± S.E.

III.3 Discussion

This study demonstrated that differentiating HC11 cells using DIP treatment up-regulated their secretion of IGFBP-5 by up to 10-fold and that, during this process, IGFBP-2 secretion was down regulated. The increase of IGFBP-5 expression during differentiation has also been shown in Schwann cells (Cheng et al., 1999), mouse osteosarcoma cells (Schneider et al., 2001), and mouse C2 myoblast cells, where IGFBP-5 is the only IGFBP produced by terminally differentiating cells (James et al., 1993; Rotwein et al., 1995). However, later work from the same group (James et al., 1996) also showed that the over-expression of IGFBP-5 in transfected C2 myoblast cells led to an inhibition of cellular differentiation. This suggests a potential dual nature of IGFBP-5 action such that during the differentiation process it is upregulated, whereas at high levels, it acts as an inhibitor of differentiation. In agreement with this concept, it has been shown that typical augmentation of IGF actions takes place when IGFBP and IGF are approximately equimolar, but when IGFBP concentrations are in excess, IGF actions are inhibited (Ewton et al., 1998). It appears that the apoptotic action of IGFBP-5 in the mammary gland *in vivo* is dependent upon the very high concentrations of binding protein that accumulate in the involuting gland (50 mg/l), which is several orders of magnitude greater than that of IGF-1 (Tonner et al., 2002). In addition, although no proteolytic activity towards ^{125}I-labelled IGFBP-2 or -5 was observed in medium conditioned by either undifferentiated or differentiated HC11 cells (data no shown), western blots of IGFBP-5 in HC11 conditioned medium clearly indicated immunoreactive IGFBP-5 fragments. It is possible that IGFBP-5 is proteolysed by an enzyme activity present on the surface of HC11 cells or by a protease(s) secreted by HC11 cells and which may be associated with the extracellular matrix.

Down regulation of IGFBP-2 during differentiation has been reported during the retinoic acid (RA)-induced differentiation of the human neuroblastoma cell line SK-N-BE(2) (Bernardini et al., 1994), in the C2C12 mouse myoblast cell line (Ernst et al., 1992) and in the human colon adenocarcinoma cell line CaCo-2 (Zhang et al., 1995). Whether the down regulation of IGFBP-2 during the process of cell differentiation is an important mechanistic feature of this process remains unknown. However, analysis of IGFBP-2 levels in HC11 cell conditioned medium indicated that dexamethasone was inhibitory for IGFBP-2 secretion and that prolactin stimulated the secretion of

this protein (Figure III. 5). Therefore, the down regulation of IGFBP-2 which is seen on differentiation of HC11 cell cultures appears predominantly to be a negative regulatory effect of dexamethasone, which is dominant over the stimulatory action of prolactin on IGFBP-2 levels.

The fact that substantial quantities of IGFBP-5 were secreted on day 6 of culture in undifferentiated HC11 cells (Figure III.2) indicates that differentiation and IGFBP-5 secretion profiles can be disconnected. In addition, analysis of the contribution of each of the hormones D, I and P to the regulation of IGFBP-2 and-5 secretion (Figure III. 5 and 6) clearly showed that up-regulation of IGFBP-5 can be dissociated from the process of HC11 cell differentiation, as well as the levels of IGFBP-2 secretion. Although IGFBP-5 is upregulated during differentiation of cells induced by treatment with the DIP lactogenic hormone mix, binary combinations of these hormones also increased levels of IGFBP-5, but did not stimulate the synthesis of the differentiation marker β-casein. In addition, the ability of non-competent HC11 cells to up-regulate IGFBP-5 expression following DIP treatment and the continued up-regulation of IGFBP-5 levels in the concurrent presence of EGF during DIP-induced differentiation, supports the conclusion that IGFBP-5 regulation is dissociated from HC11 cell differentiation (Phillips et al., 2003). It also appears that the secretion of IGFBP-2 is regulated by an inhibitory effect of dexamethasone and a stimulatory effect of prolactin and is not greatly affected by cell differentiation itself. Nonetheless, for both IGFBPs there is a clear dissociation between the process of cell differentiation and the regulation of IGFBP accumulation in conditioned medium.

HC11 cells have been used extensively as a model of mammary differentiation since they can be induced to differentiate sufficiently to express β-casein, a classical marker of mammary epithelial cells. However, it should be noted that they express β-casein at extremely low levels compared with the normal mammary gland and they do not synthesise other caseins characteristically present in milk. Thus they probably represent the earliest stages of differentiation, possibly limited by their inability to secrete caseins to any great extent. In spite of these limitations, they served as a useful comparison for the changes occurring between day 18 of pregnancy and day 1 of lactation, which DIP treatment of HC11 cells attempts to mimic.

The mRNA expression profile of IGFBPs was also demonstrated using quantitative RT-PCR to examine similarities and differences in IGFBP mRNA expression profiles between mammary gland and the HC11 cell line. Both HC11 cells and mammary tissue expressed very low levels of IGFBP-1, indicating that this binding protein does not play a major role in the mammary gland at this time. Although IGFBP-2 was expressed at similar levels in HC11 and mammary tissue, the expression profile of this IGFBP went in opposite directions during DIP treatment of HC11 cells and differentiation of the mammary gland. However, IGFBP-5 mRNA expression was increased during differentiation of HC11 cells, which is consistent with its up-regulation during the differentiation of epithelial cells that occurs *in vivo* during lactogenesis in the mammary gland. IGFBP-3 expression was the highest in both the mammary gland and HC11 cells and had the most consistent levels of expression during the different developmental stages in the gland. This suggests that in spite of the fact that IGFBP-3 is the major binding protein in serum, significant amount of IGFBP-3 was required in the mammary gland locally. In addition, *in situ* hybridisation work has shown that IGFBP-3 transcripts localise to epithelial cells in the mouse mammary gland (Wood et al., 2000). However, although significant levels of IGFBP-3 mRNA were detected in HC11 cells by quantitative RT-PCR, secretion of IGFBP-3 protein from either undifferentiated or differentiated HC11 cells was undetectable (data not shown). There is the possibility that IGFBP-3 is regulated at the level of translation in HC11 cells or that the protein is subject to proteolysis, but clearly this remains an important area for investigation. Interestingly, the mouse mammary epithelial cell line (COMMA-D/MME) has been shown to secrete IGFBP-3 (Skaar and Baumrucker, 1993).

In contrast to IGFBP-3, IGFBP-5 expression appeared to be highly regulated during mammary gland development. *In situ* hybridisation analysis has also shown IGFBP-5 transcripts are localised to epithelial cells in mouse and rat mammary glands (Rosato et al., 2002; Tonner et al., 1997; Wood et al., 2000). IGFBP-5 mRNA expression was significantly increased (54-fold) between day 10 of lactation and day 2 of involution and at least an order of magnitude greater expression level than any of the other IGFBPs in the other developmental stages (Figure III. 3). This verifies the previous finding of high concentrations of IGFBP-5 in the mammary gland during

involution in the rat (Tonner et al., 1997), and demonstrates that this up-regulation of IGFBP-5 operates at either the level of transcriptional control or message stability. IGFBP-5 mRNA expression during differentiation of HC11 cells by DIP treatment was in agreement with western blot data for IGFBP-5 protein levels. Although IGFBP-5 may act to inhibit the survival effects of IGFs, it is important to recognise that IGFBP-5 may also induce cell death in an IGF-independent manner. This is supported by recent work from others, where the ability of IGFBP-5 to inhibit the growth of human breast cancer cells both *in vitro* and *in vivo* was shown to be via an IGF-independent effect of the binding protein (Butt et al., 2003). HC11 cells did not express IGFBP-4 or -6, whilst both were expressed in mammary tissue, suggesting the stroma as the main source.

In conclusion, we have demonstrated that the IGFBP profile in the mouse mammary epithelial HC11 cell line is independent of the state of cellular differentiation. Whether HC11 cells up-regulate IGFBP-5 secretion under conditions of apoptosis as well as during cellular differentiation in this in vitro model, is the subject of further investigation. As IGFBP-5 expression has been shown to be influenced in HC11 cells by DIP treatment, we concluded that this provides an extremely useful model in which to study the potential molecular signals responsible for the induction of IGFBP-5 expression in the involuting mammary gland (See Chapter IV). It is also clearly of interest to determine whether IGFBP-5 is directly apoptotic on mammary epithelial cells *in vitro* as it appears to be *in vivo* (Tonner et al., 2000a; Tonner et al., 2002).

Chapter IV. Regulation of IGFBP-5 in HC11 cells

IV.1 Introduction

Our laboratory has been studying IGFBP-5, a 252-amino acid protein that is the most conserved of the six IGFBPs (James et al., 1993). The sequence and exon-intron structure of the IGFBP5 gene is highly conserved in human, mouse and rat. It consists of four exons and is located adjacent to IGFBP-2 on chromosome I in mice (Kou et al., 1994a; Kou et al., 1994b) and on chromosome II in humans (Allander et al., 1994). The first intron, which encompasses more than 50% of the gene, is several kilobases in length. The promoter region has a simple structure, with a 37-bp segment of the proximal sequence almost completely conserved in the rat and human genes, and which has been shown to control basal transcription (Allander et al., 1994; Zhu et al., 1993). Conserved TATAA and CAAT consensus sequences are present upstream of the transcription start in human (Allander et al., 1994), mouse (Kou et al., 1995) and rat (Zhu et al., 1993).

HC11 cells have been used extensively as a model of mammary differentiation. We have carried out an *in vitro* study in these cells that parallels our previous *in vivo* study of IGFBP-5 (Tonner et al., 2002). HC11 cells undergo proliferation and differentiation similar to that observed *in vivo* during mammary gland development and can be induced to differentiate and express β-casein by treatment with dexamethasone, insulin and prolactin (DIP) (Ball et al., 1988a; Ball et al., 1988b). As discussed in Chapter III, we have demonstrated that DIP treatment of HC11 cells increased IGFBP-5 protein expression levels by up to 10-fold (Phillips et al., 2003). More recently we have shown that these increases in IGFBP-5 protein levels also correlated with a significant increase in the level of IGFBP-5 messenger RNA in HC11 cells treated with DIP (Boutinaud et al., 2004). Therefore, for analysis of promoter function we chose the HC11 cell line, as this could provide an *in vitro* system where IGFBP-5 expression could be quickly up-regulated.

The aim of this study is to investigate the regulation of IGFBP-5 expression in HC11 cells. To investigate the regulation of IGFBP-5 through *in vitro* models, we have used transient gene

transfer studies. We used a IGFBP-5 promoter deletion series linked to the luciferase gene for the transfection of undifferentiated and differentiated HC11 cells.

IV.2 Results

IV.2.1 Comparison of transfection reagents

Gene reporter systems play an important role in the study of gene expression and regulation. Lipid-mediated transfection reagents can be used for simple and reproducible delivery of foreign DNA into mammalian cells and surpass other types of transfection reagents because they are easy to use, gentle to cells and provide consistent reproducible results (Ausubel et al., 1995). However, among cell types, lipid-mediated transfection reagents can differ in transfection efficiency and proper dosage. Moreover, the amount of transfection reagent that can be used is limited by cytotoxic effects of either the lipid or the expressed plasmid protein. Therefore, the first step to achieving high transfection efficiencies was to choose the regents and to optimise the combination of lipid and DNA for the HC11 cell line.

In this initial experiment, to find the most efficient transfection reagent and to optimise condition for transfection into the HC11 cells, the efficacy of the various transfection reagents were compared using the pGL3-Control vector plasmid (Promega), which contained the SV40 promoter, enhancer and the fire fly luciferase gene as a reporter. Tfx (Promega), SuperFect (Quiagen), GeneJuice (Novagen) and FuGENE 6 (Roche Applied Science) were chosen to test transfection efficiency in HC11 cells. Cells were harvested 48 hours post-transfection and measured for transient expression of the luciferase reporter gene present in the pGL3-Control vector using the luciferase assay system (Promega).

As shown in Figure IV.1, in general, the higher DNA concentration that was used, the more luciferase activity was produced and the greater the amount of transfection reagent tolerated by the cell line, the higher the transfection efficiency was achieved. The HC11 cell line seemed tolerant to GeneJuice and FuGene transfection reagents as determined by the measurement of the

luciferase activity for the various volumes used. For the highest amount (6 µl) of FuGene transfection reagent, the luciferase activity was nearly constant with the increasing amounts of DNA. Although a 2:1 ratio of lipid to 1 µg of DNA using GeneJuice had the largest luciferase activity, it also had the greatest variability (see error bars). However, the GeneJuice reagent with 1 µg of plasmid and a 3:1 ratio of GeneJuice/well clearly outperformed other transfection conditions.

In summary, our comparison of transfection reagents clearly demonstrated that GeneJuice was the most efficient in the transfection of HC11 cells. Therefore, we chose to use GeneJuice for our further experiments. The optimal DNA concentration added to the cells was 1 µg per each well with 3 µl of GeneJuice Reagent.

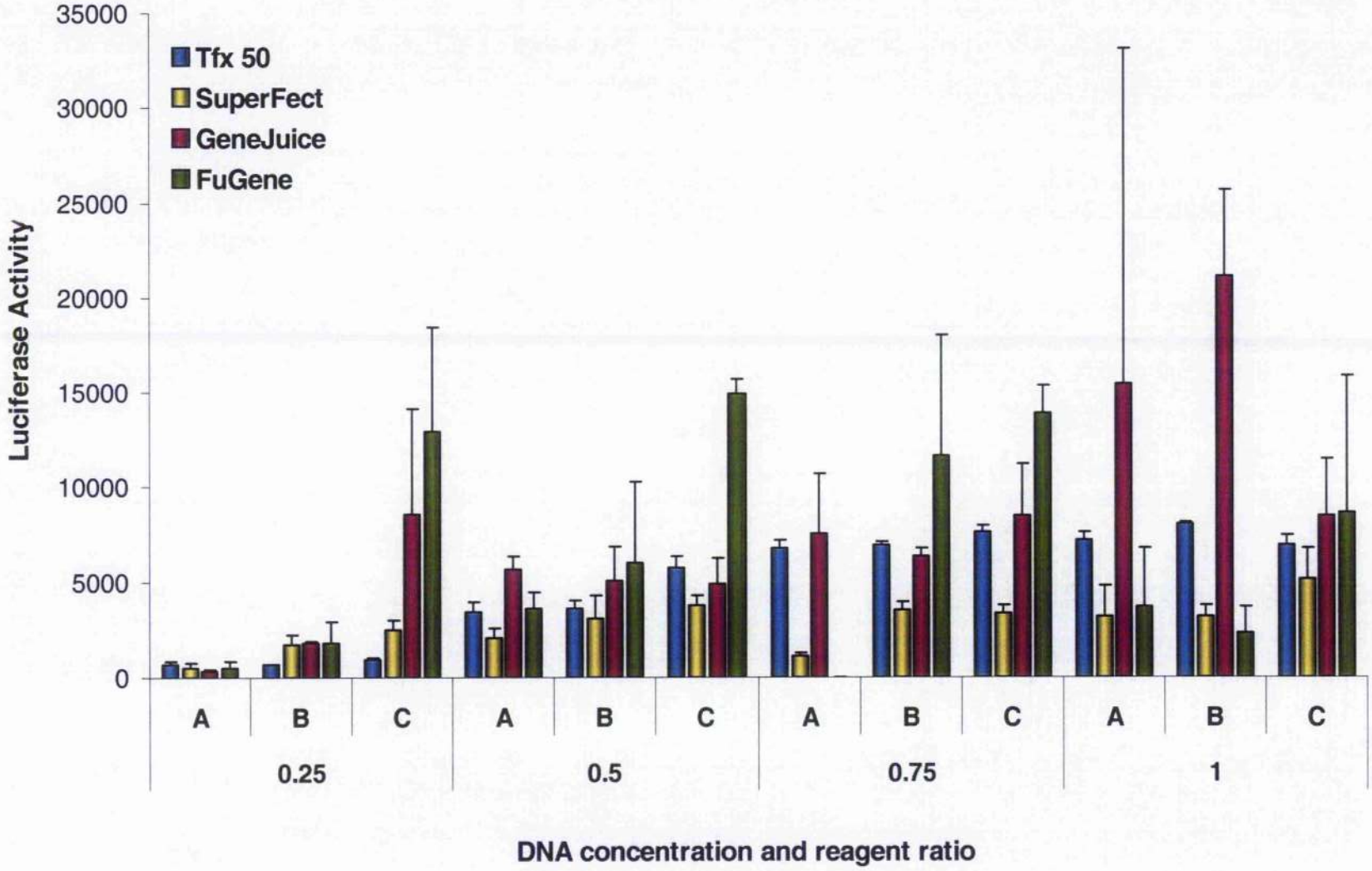

35000
30000
25000
20000
15000
10000
5000
0
Luciferase Activity
Tfx 50
SuperFect
GeneJuice
FuGene
A
B
C
A
B
C
A
B
C
A
B
C
0.25
0.5
0.75
1
DNA concentration and reagent ratio

Figure IV.1 Luciferase activity comparison between different transfection reagents. HC11 cells were plated at a density approximating 50 % confluence ($2.5x10^4$/well) in 24 well plates before 48 hr of transfection. Transfections were performed with various amounts of DNA (0.25, 0.5, 0.75, 1μg) and various ratios of transfection reagents to DNA. A) 2:1, except FuGene; 2:3, B) 3:1, C) 5:1 for SuperFect, GeneJuice, 4:1 for Tfx-50 and 6:1 for FuGene using the manufacturer's protocol. Transfected cells were maintained in culture for a further 48 hr in complete medium. Cell extracts were prepared and assayed for luciferase with the Promega Luciferase Assay System.

IV.2.2 IGFBP-5 upregulation

IV.2.2.1 IGFBP5 promoter-luciferase gene constructs

Mouse IGFBP-5 promoter – luciferase reporter constructs: IGFBP5-Luc2, 3, 4 and 5 were kindly provided from Peter Rotwein (Oregon Health & Sciences University, USA). The structure of the IGFBP-5 promoter deletion series linked to the luciferase reporter gene is shown in Figure IV.2. Briefly, these contain different fragments of the mouse IGFBP-5 5' flanking DNA ranging from –3000 to –156 at the 5' end: Luc2 extends to the BamHI site (-3000), Luc3 to EcoRI (-1406), Luc4 to PstI (-1004) and Luc5 to StuI (-156), with all constructs containing the same 3' end point at + 120 bp in exon 1. All of the series have been blunt end cloned into the blunted HindIII site of the pGL2-basic vector (Promega) (Kou et al., 1994a). To confirm each construct, restriction enzyme analysis was performed and all the constructs were sequenced by MWG BioTech.

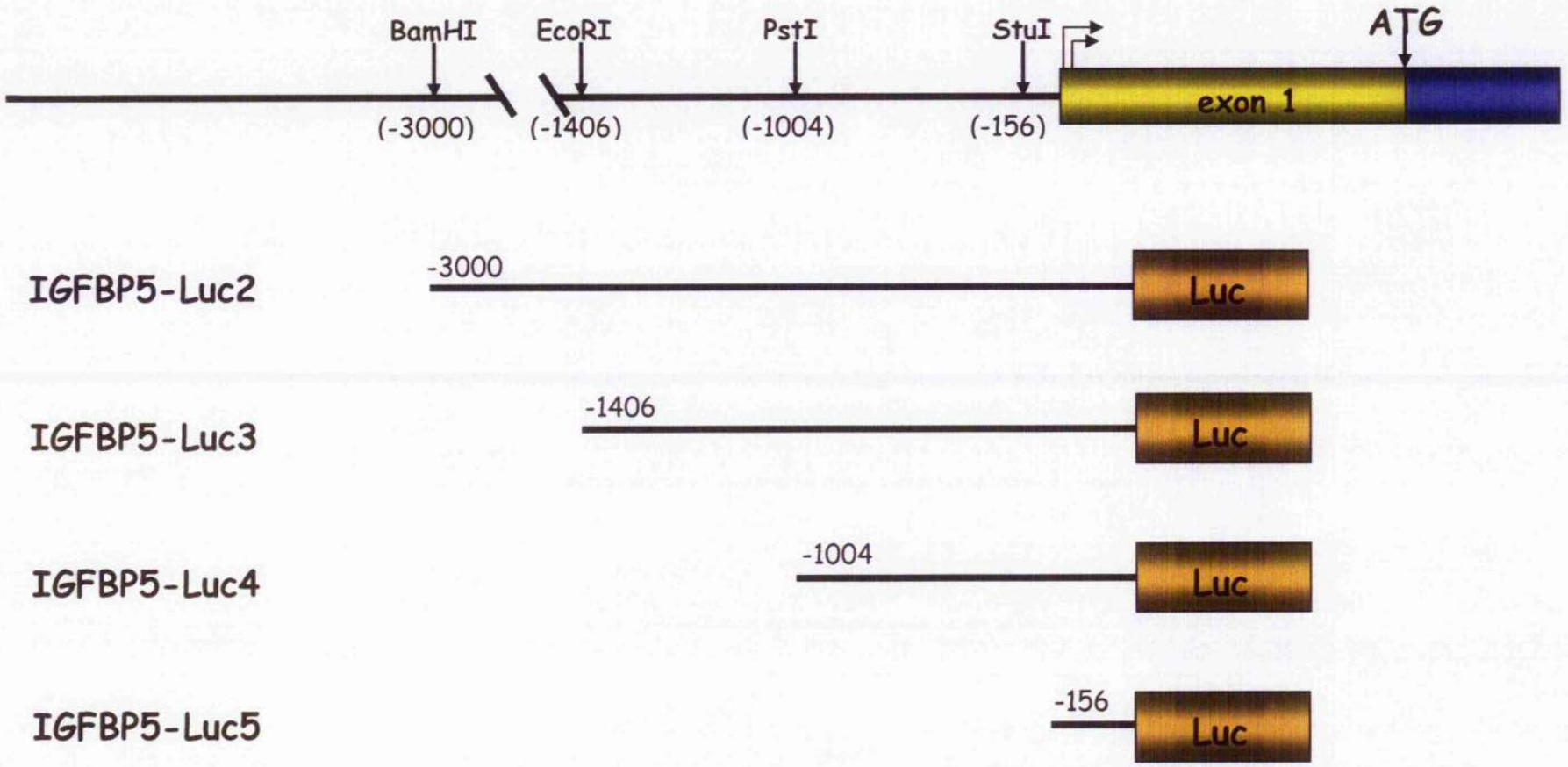


Figure IV.2 Promoter deletion series of chimeric IGFBP-5-Luciferase reporter gene constructs. Different fragments of mouse IGFBP-5 5'-flanking DNA ranging from –3000 to –156 with respect to the first transcription start site, all containing the same 3' end point at +120 bp (Kou et al., 1994b), were cloned 5' to a luciferase reporter gene (Kou et al., 1995).

IV.2.2.2 IGFBP-5 luciferase reporter constructs are inducible by DIP treatment

The results described above in Chapter III indicate that IGFBP-5 protein and mRNA levels increase during HC11 cell differentiation using DIP treatment, and western blots (Phillips et al., 2003) and quantitative PCR results (Boutinaud et al., 2004) confirming this have been published previously. Accordingly, we next sought to identify the promoter regions mediating this effect. To investigate hormonal induction of IGFBP-5, we transfected four different promoter deletion constructs (IGFBP5-Luc2, 3, 4 and 5, see Figure IV.2) into HC11 cells in the presence or absence of DIP (Figure IV.3).

Interestingly, luciferase activities from HC11cells transfected with all constructs in the presence of DIP were clearly significantly higher than those in non-treated cells (Figure IV.3). This demonstrated that all four-deletion series constructs containing between 3000 bp of 5'-flanking region and 120 bp of IGFBP-5 gene were inducible by DIP treatment. However we found the strongest activity was with the IGFBP5-Luc4 construct, a fusion plasmid containing 1004 base pairs of 5'flanking DNA and the initial 120 nucleotides of exon 1, in both undifferentiated (without DIP) and differentiated (with DIP) HC11 cells. The luciferase activity directed by IGFBP5-Luc4 was stimulated 105-fold with DIP treatment (Figure IV.4). In contrast, the luciferase activity of the largest construct, IGFBP5-Luc2 (containing 3000 bp of 5'-flanking region and 120 bp of exon 1 of the IGFBP-5 gene) was the lowest in the presence and absence of DIP, demonstrating approximately a 20-fold induction upon DIP treatment.

Therefore, the deletion of 1594 bp of 5'-flanking DNA going from IGFBP-Luc2 to IGFBP5-Luc3 resulted in an increase in the fold-induction of luciferase activity upon DIP treatment from 20 to 28-fold, and a further 402 bp deletion to produce IGFBP5-Luc4 produced the optimum 105-fold induction. Furthermore, an additional deletion of 848 bp of 5' sequence to IGFBP5-Luc5 led to a marked decrease in promoter function, demonstrating only a 30-fold induction upon DIP treatment.

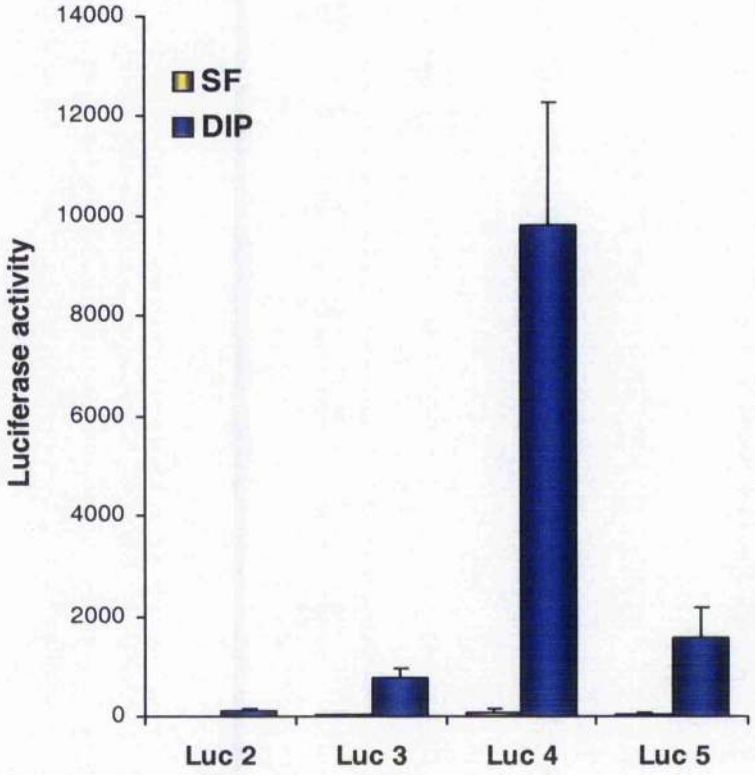


Figure IV.3 Analysis of promoter activity of the mouse IGFBP-5 promoter-luciferase deletion series constructs after transfection into HC11 cells in the presence and absence of DIP. HC11 cells were transiently transfected with 1 μg of IGFBP-5 promoter deletion series construct per each well after 2 days of incubation with Insulin and EGF. On the same day of transfection, cells were cultured either with or without DIP. Cells were harvested 48 hr after transfection and transfection efficiencies were normalised determining the relative transfection efficiencies by filter hybridisation with ^{32}P-labelled pGL3-basic vector. Results are means ± S.E.M. of triplicate using duplicate preparations of plasmid DNA. The result is representative of studies which were conducted on at least 5 occasions.

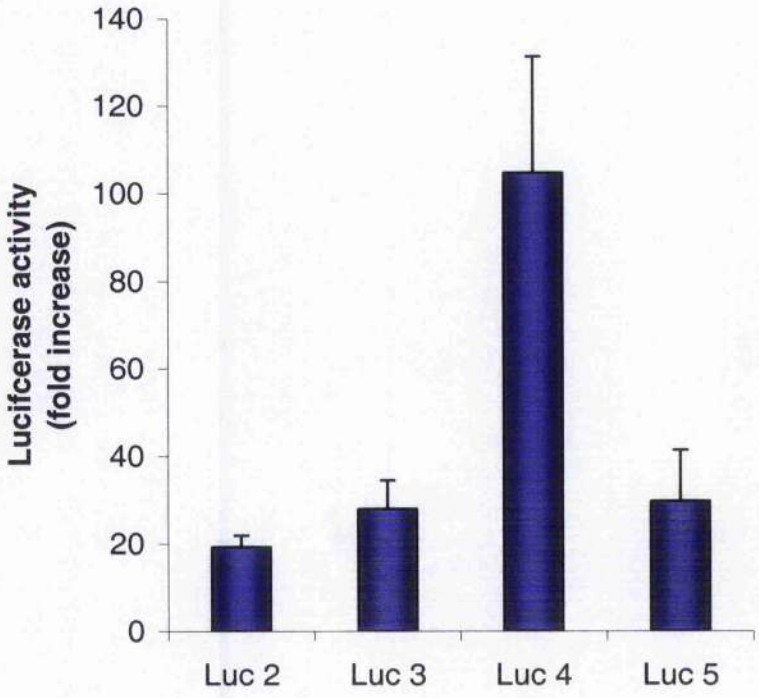


Figure IV.4 Fold Induction of Luciferase activity by DIP Treatment. The fold induction by DIP treatment was calculated by dividing the 48 hr post-transfection luciferase activities from the HC11 cells in the presence of DIP by the activities from transfected cells in the absence of DIP.

IV.2.2.3 Luciferase activity comparison: pGL2- vs pGL3- control vector vs IGFBP5-Luc4 construct

To this point, we had used the pGL3-control vector as an efficiency of transfection and luciferase activity control for each transfection. Since the pGL3-control vector has its own very powerful promoter and enhancer, we were never able to achieve the same high levels of luciferase activity with IGFBP-5 promoter series. However, the pGL3-vector series is also a much more advanced vector system than the pGL2-vector, in which the IGFBP-5 promoter series was originally cloned. The backbone of the pGL2 Luciferase Reporter Vectors was re-designed for the pGL3 Vectors for increased expression, and contains a modified coding region for firefly (*Photinus pyralis*) luciferase that has been optimised for monitoring transcriptional activity in transfected eukaryotic cells. These changes were made to increase sensitivity of the Luciferase Assay System, facilitate the use of the vectors, and reduce unanticipated interactions between the plasmid backbone and cellular factors. Modifications include the substitution of C-terminal amino acids which results in cytoplasmic localization of luciferase and increased signal in the Luciferase Assay System (See detail in Promega pGL3 Luciferase Assay System). Thus, initially we decided to compare the luciferase activity between pGL2- and pGL3- control vectors (Figure IV.5). IGFBP5-Luc4 construct, which has the highest luciferase activity among the IGFBP-5 promoter deletion series, was also included in this experiment. We transfected pGL2-, pGL3-control vector and IGFBP5-Luc4 constructs into HC11 cells using the same protocol for transfection and DIP treatment as was used for the IGFBP-5 promoter described above. The luciferase activity from the cells transiently transfected with pGL3-control vector was considerably higher than that of both the pGL2-control vector or IGFBP5-Luc4 (although we note that the luciferase activity from IGFBP5-Luc4 in the pGL-2 basic vector plasmid had a similar or even greater activity than that from the pGL2-control vector). Therefore, in order to optimise our transfection-luciferase studies further, we decided that it was worthwhile to sub-clone the IGFBP-5 promoter series into the pGL3-basic vector.

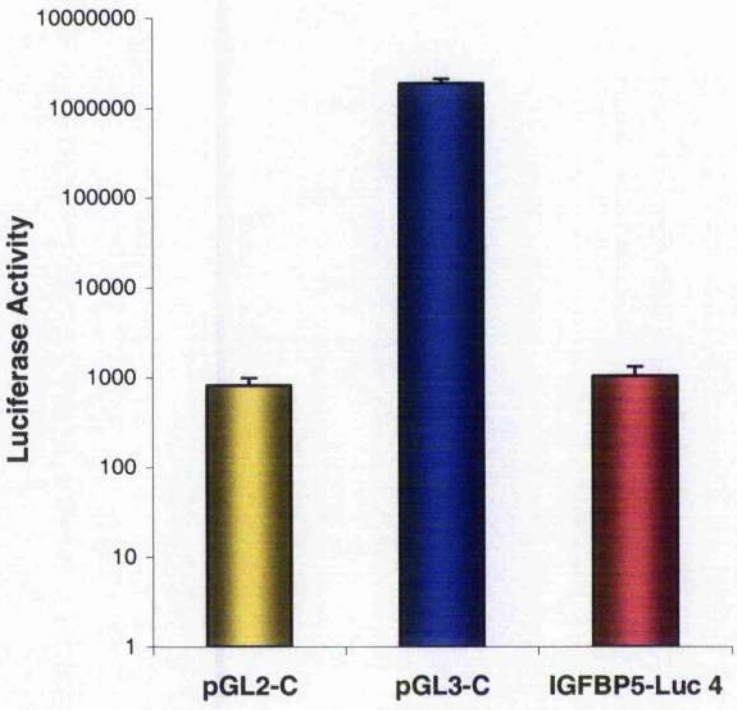


Figure IV.5 Luciferase activity comparison between pGL-2, pGL-3 Control vectors and IGFBP5-Luc4. HC11 cells were transiently transfected with 1 μg of pGL2-, pGL3- control vector (Promega) and IGFBP5-Luc4 construct. Transfections were performed in the presence of DIP. Cells were harvested 48 hr after transfection and assayed for luciferase activity. Data were normalised against the amount of plasmid DNA taken up by the cells. Result is mean ± S.E.M. of triplicate. The y-axis is in logarithmic scale.

IV.2.2.4 Subcloning into pGL3-basic vector

To subclone all of the IGFBP-5 promoter deletion series into the pGL3-basic vector, IGFBP5-Luc2, 3, 4 and 5 constructs in pGL2-basic vector were initially digested with XhoI and NarI restriction enzymes. The resulting XhoI and NarI fragments were then cloned into the same sites in the pGL3-basic vector (Promega) as described in Materials and Methods. To confirm the constructs in pGL3-basic vector were correct, enzyme analyses were performed.

IV.2.2.5 IGFBP-5 promoter-pGL3 activity analysis in HC11 cells

The IGFBP-5 promoter deletion series constructs, now cloned in the pGL3-basic luciferase reporter vector were transiently transfected into the HC11 cell line as described above. In the literature and from the studies with the IGFBP-5 promoter deletion series in the pGL2 vector above (Figure IV.4), there was again very little luciferase activation with the smallest construct, IGFBP5-Luc5 (Figure IV.6). A previous study using Hep G2 cells has shown that transfection of IGFBP5-Luc4 stimulated the highest level of luciferase activity (Kou et al., 1995), and our results in HC11 cells would agree with that. This activity went back down again with the larger members of the promoter series, IGFBP5-Luc3 and IGFBP5-Luc2.

Therefore, the pattern of luciferase activity for the IGFBP-5 promoter deletion series in either pGL-2 or pGL-3 plasmids was largely the same. Overall, this would imply that there is a transcriptional activation site/s between positions –1004 to –156 in the IGFBP-5 promoter to account for the difference between IGFBP-Luc4 and IGFBP-Luc5 and furthermore, downstream repressor elements between –3000 to –1004 bp to explain the activities in IGFBP-Luc2 and IGFBP-Luc 3 being lower than IGFBP-Luc4.

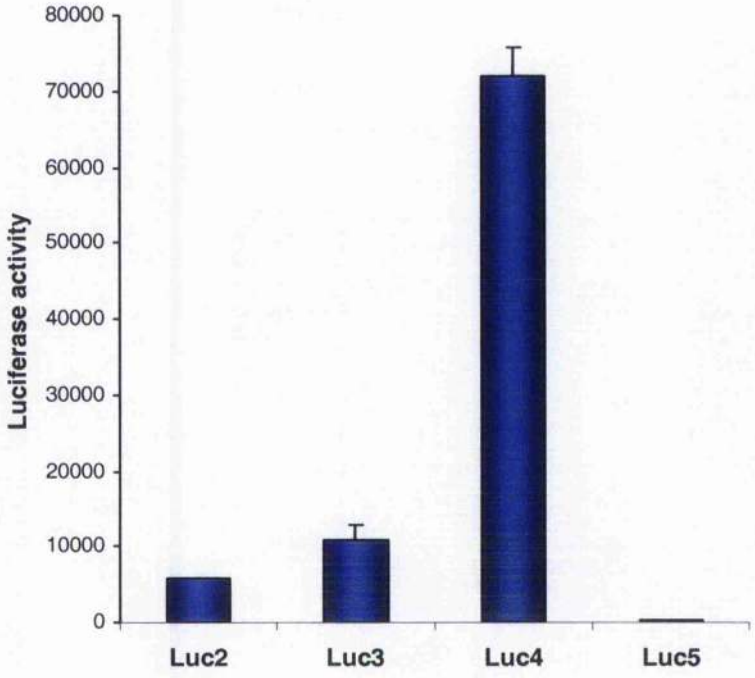


Figure IV.6 Identification of luciferase activity in the IGFBP-5 promoter deletion series in the pGL-3 vector following DIP treatment of HC11 cells. HC11 cells were transiently transfected with 1 μg of deletion series of IGFBP-5 promoter-luciferase reporter gene construct. Cells were harvested 48 hr after transfection and transfection efficiencies determined by filter hybridisation with ^{32}P-labelled pGL3-basic vector. Transfections were performed in the presence of DIP. Results are means ± S.E.M. of triplicate experiments using duplicate preparations of plasmid DNA. The result is representative of studies conducted on three occasions.

IV.2.3 Specification of Regulatory Site/s Position/s

IV.2.3.1 Construction of Luc4B/Luc4(XhoI)

In an attempt to localise the regulatory elements in the IGFBP-5 promoter responsible for the effects reported above, we decided to modify the IGFBP-5 promoter deletion series by making further deletions. We knew that the enhancer element responsible for the high activity of IGFBP5-Luc4 must lie between positions −1004 and −156 bp, so we set out to make a new deletion construct derived from IGFBP5-Luc4 (named IGFBP5-Luc4B), in which this region would essentially be halved. The structure of the new constructs and the strategy for making them is shown in Figure IV.7. To create IGFBP5-Luc4B, site-directed mutagenesis was performed at position −556 on the IGFBP5-Luc4 construct using the QuickChange Site-directed system (Stratagene) as described in chapter II. This mutagenesis introduced a unique XhoI restriction enzyme site, and the intermediate construct containing this mutation was named IGFBP5-Luc4(XhoI). Restriction enzyme analysis confirmed the presence of the new XhoI site in the IGFBP5-Luc4(XhoI) construct (Figure IV.8A).

Following this, 448 bp of sequence was deleted from the 5' end of IGFBP5-Luc4(XhoI) by simply digesting with XhoI, purifying the upper plasmid band and re-ligating the XhoI site in the promoter to the unique XhoI site in the polylinker sequence of the plasmid. This generated IGFBP5-Luc4B, which now ends at position −556 bp of 5'-flanking region of IGFBP-5 promoter. This was confirmed by a SacI restriction digest of IGFBP5-Luc4, IGFBP5-Luc4(XhoI) and IGFBP5-Luc4B, which clearly shows the smaller size of promoter region in IGFBP-Luc4B (Figure IV.8B).

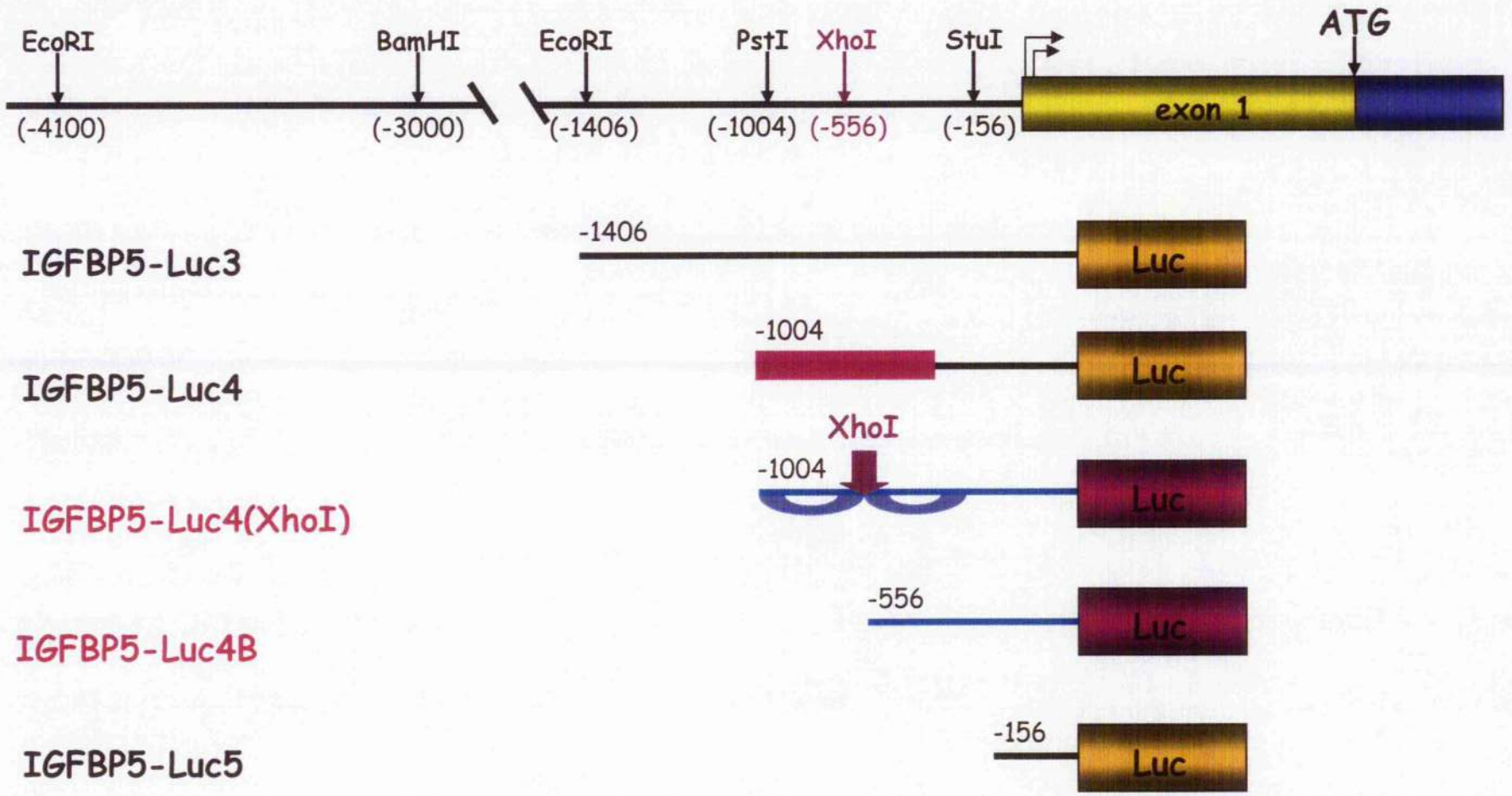


Figure IV.7 Mutant IGFBP-5 promoter constructs Luc4(XhoI) and Luc4B.

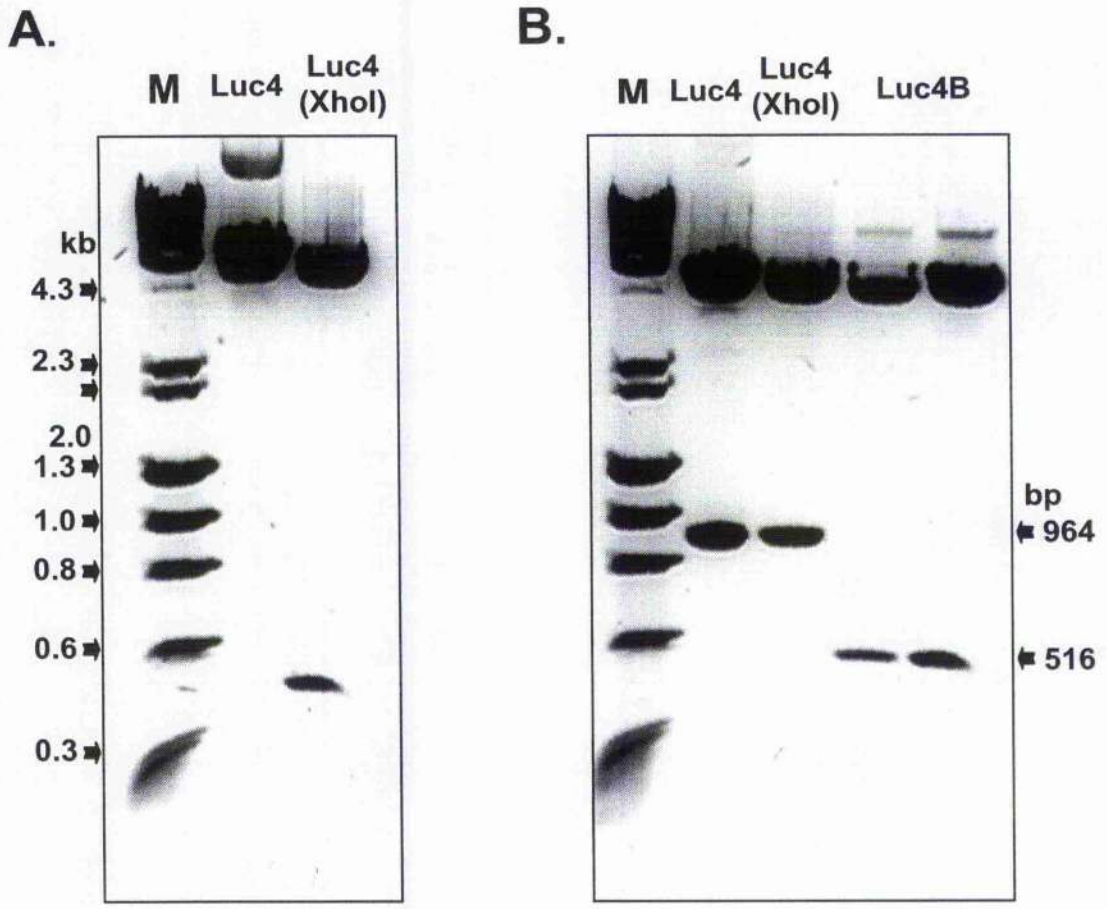


Figure IV.8 Restriction enzyme analyses of mutant IGFBP5-Luc4(XhoI) and Luc4B constructs in pGL3-basic vector. A. XhoI digests of IGFBP5-Luc4, IGFBP5-Luc4(XhoI) constructs. **B.** SacI. digests of IGFBP5-Luc4, IGFBP5-Luc4(XhoI) and Luc4B constructs. M represents DNA size markers (λ-HindIII and $\phi\chi174$).

IV.2.3.2 Response of IGFBP5-Luc4(XhoI) and IGFBP5-Luc4B in HC11 cells in the presence of DIP

The results of multiple transfection experiments are shown in Figure IV.9. The highest luciferase activity was found with IGFBP5-Luc4. Interestingly, we found that there was a 35% reduction in luciferase activity with IGFBP5-Luc4B relative to IGFBP5-Luc4, which immediately tells us that some enhancing activity must reside within the 448bp between positions –1004 and –156 in the IGFBP-5 promoter. However, the activity of IGFBP5-Luc4B was still higher than IGFBP5-Luc5, indicating that there must be multiple enhancer sites between positions –1004 and –156 and residing on either side of position –556.

More unexpectedly, we also found that there was a 22% reduction in activity with the IGFBP5-Luc4(XhoI) construct relative to IGFBP5-Luc4 (Figure IV.9). It is possible that the substitution of three base pairs at position –556 in IGFBP5-Luc4(XhoI) (ggg to ctc; see Figure IV.11) has interrupted an important transcriptional binding site. Therefore, we decided to search for candidate transcriptional binding sites at this location using the MatInspector Programme (Genomatix, Munich, Germany).

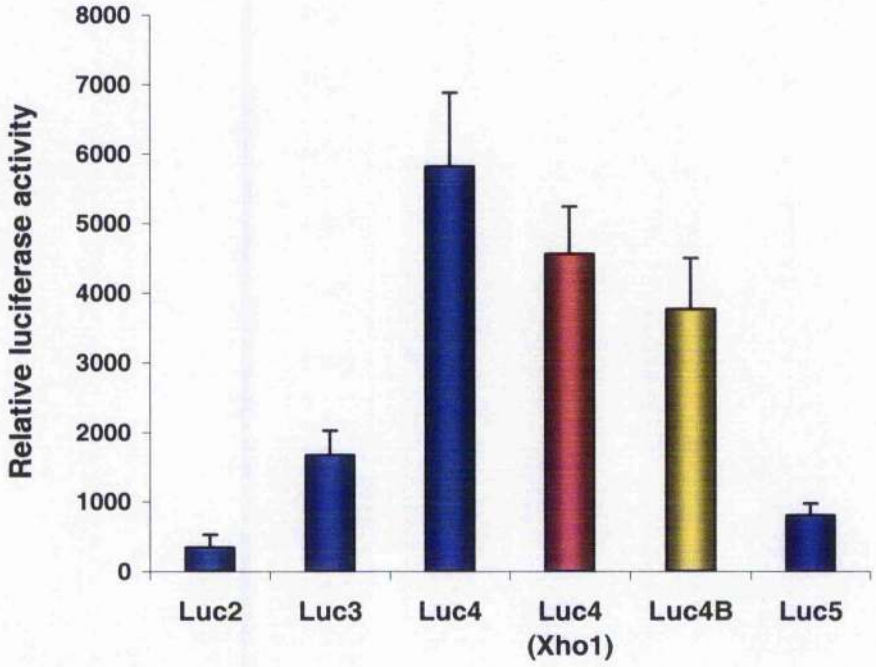


Figure IV.9 Promoter deletion analysis of the IGFBP-5 gene. Luciferase activity was determined after transfection, as described in Materials and Methods. Data represent the mean of five to eight experiments, each in duplicate experiments using duplicate or triplicate plasmid DNA preparations ± standard error of the mean. Each experiment was normalised for relative transfection efficiencies by filter hybridisation with ^{32}P-labelled pGL3-basic vector and also against the luciferase activity of pGL3-control vector.

IV.2.3.3 Potential regulatory elements at position –556 within the IGFBP5 promoter

Computer analysis using the MatInspector programme of the primer which created the XhoI site in the IGFBP5-Luc4(XhoI) indicated four potential transcriptional sites, shown in Figure IV.10. These were sites for Hox-1.3 (a vertebrate homeobox gene), a GC box element known as SP1F, a zinc finger transcriptional factor (ZBP-89) and the MYC-associated zinc finger protein related transcriptional factor. However, our substitutions only hit the "core" base pairs (most highly conserved, shown in upper case in Figure IV.10) in the sites for the GC box and ZBP-89 which appears to play an important role in intestinal cell proliferation, differentiation, and oncogenesis through p53 (Bai et al., 2004; Chen et al., 2003; Merchant et al., 1996; Remington et al., 1997). This transcriptional factor could act as either an activator or repressor depending upon the target promoter, for examples, it represses vimentin (Yamagishi et al., 1994) whereas activates the STAT-1 (Bai and Merchant, 2003). Therefore, if ZBP-89 is one of the transcriptional factor interrupted by mutant, it would act as an activator of IGFBP-5 promoter and induce differentiation and aoptosis.

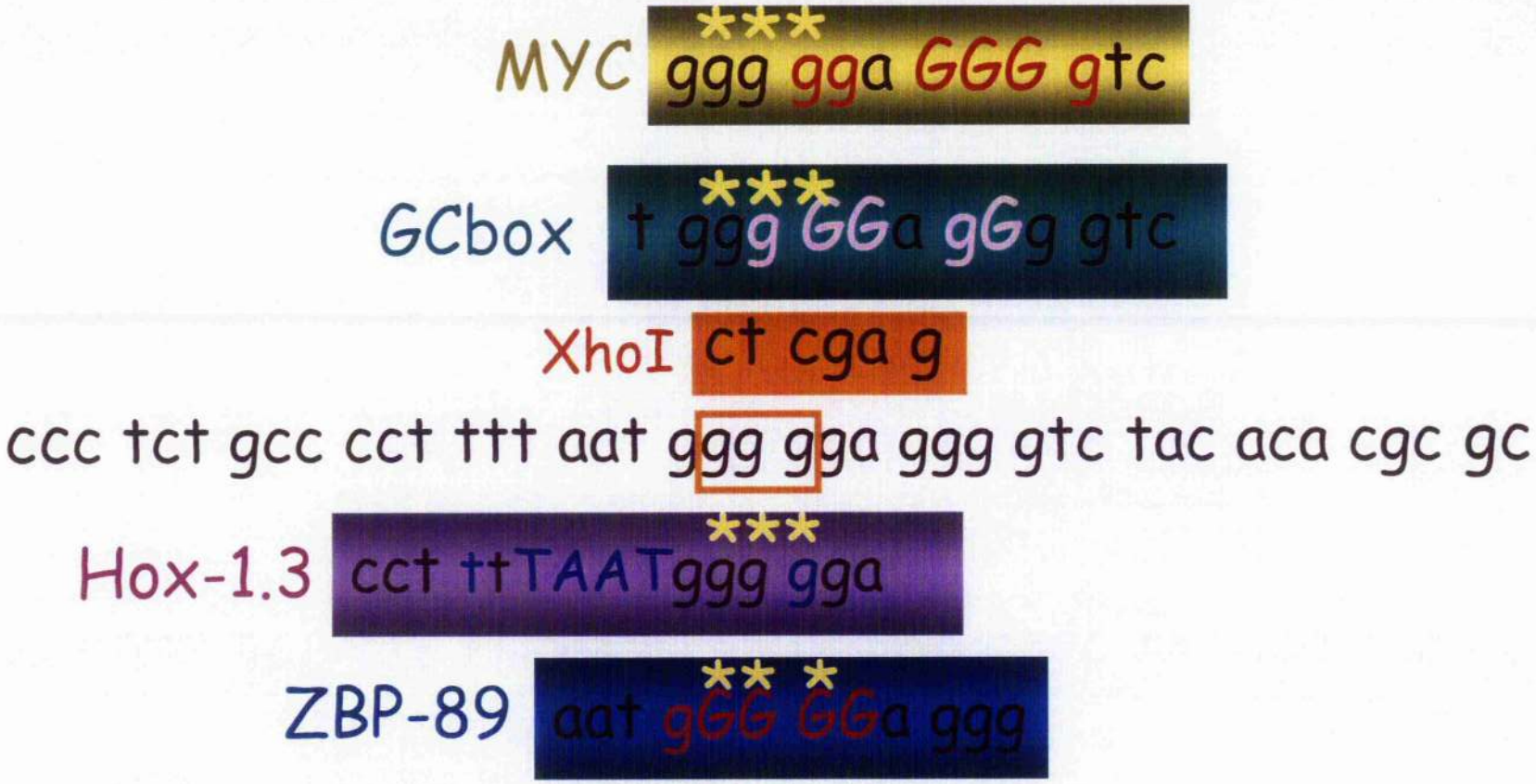

MYC
***
ggg gga GGG gtc
GCbox
***
t ggg GGa gGg gtc
XhoI
ct cga g
ccc tct gcc cct ttt aat ggg gga ggg gtc tac aca cgc gc
Hox-1.3
***
cct ttTAATggg gga
ZBP-89
** *
aat gGG GGa ggg

Figure IV.10 MatInspector sites potentially disrupted by the XhoI site substitutions. The primer sequence which was used to make IGFBP5-Luc4(XhoI) is shown in the middle the substituted sequence (boxed) is indicated. These were altered to create the XhoI restriction enzyme site shown above. The consensus binding sites for four different transcription factor candidates are indicated on either side; HOX-1.3, GC box SP1F, zinc finger transcriptional factor ZBP-89 and MYC-associated zinc finger protein related transcriptional factor. Coloured letters in each transcription factor candidate's box represent highly conserved sequences and capital letters denote core sequences. Yellow asterisks indicate mutated sequences in each consensus site.

IV.3 Discussion

In this study using transient gene transfer methods, we have carried out an initial analysis of the activity of the mouse IGFBP-5 gene promoter from positions -156 to -3000 in differentiated (DIP treated) HC11 cells.

We employed a series of IGFBP-5 promoter deletion constructs spanning +120 bp of exon 1 to -3000 bp from the 5' flanking region of the mouse IGFBP-5 gene upstream of the luciferase reporter gene. HC11 cells were chosen as they undergo differentiation upon treatment with the lactogenic hormone mixture dexamethasone, insulin and prolactin (DIP) and we have previously shown that this is associated with a significant increase in the levels of both IGFBP-5 protein (Phillips et al., 2003) and mRNA (Boutinaud et al., 2004).

The IGFBP5-Luc2 construct, which has the largest amount of promoter sequence (-3000 to +120 bp), had the lowest luciferase activity compared the other shorter promoter constructs in both differentiated and undifferentiated HC11 cells. This result agrees well with the work of others (Kou et al., 1995), where they had shown a pattern of 58% activity for IGFBP-Luc2, 74% for IGFBP5-Luc3, 100% for IGFBP-Luc4 and 61% for IGFBP-Luc5 in human, primary liver cancer cell line, Hep G2. As with this previous study, we found that the strongest activity was with the IGFBP5-Luc4 construct in both undifferentiated and differentiated HC11 cells. The similarity of the pattern of activity between the different constructs in either the mammary epithelial cell line HC11 or the human primary liver cancer cell line Hep G2, suggests that the same transcriptional regulatory elements are active in both cell types and perhaps that these are used to control IGFBP-5 expression in a wide variety of cell and tissue types. Moreover, the pattern of activity across the deletion series allows us to deduce where either inhibitory or enhancing regulatory elements must lie. Our work would imply that there is a repressor element/s in IGFBP5-Luc3 (–1406 to –1004), which when deleted in IGFBP5-Luc4 results in an increase in activity, whereas there is an enhancer element/s in IGFBP5-Luc4 (between –1004 and –156), which is lost when this region is deleted in IGFBP-Luc5, thereby resulting in the vastly reduced activity seen with this latter construct.

In an attempt to narrow down the region containing the novel enhancer sequence/s, we introduced an XhoI site at position –556 and subsequently deleted –1004 to –556 of the 5' flanking region to create the IGFBP-Luc4B construct. This new construct had a lower activity than the original IGFBP-Luc4, but had a considerably higher activity than IGFBP-Luc5. This strongly suggests that there are multiple enhancer sequences between positions –1004 to –156 in the IGFBP-5 promoter, and that some of these must reside on either side of position –556. To our surprise, when we included the intermediate construct IGFBP5-Luc4(XhoI) in these experiments, we found that it too had a lower activity than IGFBP-Luc4 in differentiated HC11 cells. The only explanation that we can offer for this result is that we may have inadvertently interrupted a regulatory transcriptional binding site when we made our 3 base pair substitutions in IGFBP-Luc4(XhoI). In the light of this, we decided to search for candidate transcriptional binding sites at this location using the MatInspector Programme and this suggested four potential transcriptional sites for Hox-1.3, a GC box element known as SP1F, the zinc finger transcriptional factor (ZBP-89) and the MYC-associated zinc finger protein related transcriptional factor. None of these transcriptional factors have previously been shown to regulate the expression of IGFBP-5. In order to address whether they do have a regulatory role for IGFBP-5, future experiments could initially be carried out to mutate the critical base pairs in these sites and then look at the effects of this on luciferase activity in differentiated HC11 cells. If this were to suggest a strong candidate transcription factor, more long-term studies including DNase 1 Footprinting and Gel Shift studies could be carried out to confirm this.

To date all of the regulatory sites in the IGFBP-5 promoter, which have been shown to be active, have fallen with in the first 71bp downstream of the transcriptional start site and 70% of promoter activity was shown to reside in this region in HepG2 cells (Kou et al., 1995) (see Introduction). The important sites identified in this proximal region include an active AP-2 binding site (Duan and Clemmons, 1995) and a functional E-box (Ji et al., 1999) which controls the regulation of IGFBP-5 by the bone morphogenetic protein, BMP-7 (Hutt et al., 2000). The results presented in this chapter are important, because they demonstrate for the first time that there are other important regulatory elements further downstream in the IGFBP-5 promoter, which are active

in a mammary epithelial cell line and, therefore, by inference, may be active in the mammary gland *in vivo*. Future work merits identifying these important, as yet unknown, transcription factors.

Chapter V. Functional studies of IGFBP-5 in HC11 cells: Effects of IGFBP-5 on plasmin generation

V.1 Introduction

Our group has previously demonstrated that IGFBP-5 production by mammary epithelial cells increases dramatically during involution of the mammary gland in rodents (Tonner et al., 1997). More recently, as discussed earlier in Chapter III, using quantitative PCR, we have shown that these increases in IGFBP-5 protein level also correlated with a significant increase in the level of IGFBP-5 messenger RNA (25-fold) in involuting mammary gland (Boutinaud et al., 2004). However, this association of IGFBP-5 and apoptosis is not found in mammary gland alone. IGFBP-5 expression has also been implicated in cell death of the prostate, thyroid gland and in ovarian follicles undergoing atresia (Guenette and Tenniswood, 1994; Liu et al., 1993b; Phillips et al., 1994). A causal relationship between IGFBP-5 synthesis and apoptosis in the mammary gland has been addressed in several studies on the effects of IGFBP-5 given exogenously *in vitro* to murine mammary epithelial cells in culture (Marshman et al., 2003) and *in vivo* to mice during late pregnancy (Allan et al., 2002) as well as in a transgenic model examining the effects of over-expression of IGFBP-5 using a mammary-specific promoter (Tonner et al., 2002). *In vivo* administration of recombinant IGFBP-5 protein in late pregnancy in the mouse resulted in impaired mammary development and reduced invasion of the mammary fat pad (Allan et al., 2002). At parturition, in IGFBP-5 transgenic mice, there were increased concentrations of the pro-apoptotic molecule, caspase-3, whereas the concentration of pro-survival, Bcl-2 and Bcl-xL, were significantly reduced. DNA content in the mammary gland of transgenic mice was decreased as early as day 10 of pregnancy. In lactation, both mammary gland cell number and milk synthesis were significantly decreased (Tonner et al., 2002). Intriguingly these mice also exhibited increased concentrations of plasmin in their mammary glands. These findings suggested that a major role of IGFBP-5 in the mammary gland *in vivo* is to promote apoptosis of mammary epithelial cells

Typically, augmentation of IGF actions takes place when IGFBP and IGF are approximately equimolar, but when IGFBP concentrations are in excess, IGF actions are inhibited (Ewton et al., 1998). Our group was able to show that during involution of the mammary gland, IGFBP-5 retained its high affinity for IGF-I and concentrations in milk were in excess of 50 mg/l, which is several orders of magnitude greater than that of IGF-I. These findings strongly suggested that the increased IGFBP-5 secretion during mammary involution was acting to inhibit IGF actions and thereby induce cell death. Our group has proposed that IGFBP-5 is able to prevent the action of IGF-I possibly by sequestering IGFs to the ECM so that the cell survival factors IGF-I and -II cannot trigger cell-signalling pathways, thereby allowing the cells to undergo apoptosis (See Figure I. 9) (Flint et al., 2003; Flint et al., 2000; Tonner et al., 2002).

Recent studies have suggested that the effects of IGFBP-3 and -5 can be both IGF-dependent and IGF-independent and that interactions with particular ECM components might influence these responses (Perks et al., 2002a; Perks et al., 2002b). Various observations led us to consider whether the extremely high concentrations of IGFBP-5 in milk from involuting mammary glands were present solely to inhibit IGF actions or whether they were also involved in additional, perhaps IGF-independent actions (Flint et al., 2000).

Involution of the mammary gland includes extensive degradation of the ECM and involves extracellular proteases including the plasminogen (Pgen) system and matrix metalloproteinases (MMPs), which are involved in degrading the ECM in the later stages of tissue remodelling (Lund et al., 1996). This process is initiated by the activation of plasminogen by tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) to form plasmin (Heegaard et al., 1997b). Plasmin plays an important role in cleaving a number of pro-enzymes, MMPs, such as prostromelysins and procollagenases, and thereby initiates the degradation of the ECM at the end of lactation when extensive tissue remodelling occurs (Matrisian, 1990). High plasmin activity has been observed in the mammary gland of IGFBP-5 transgenic mouse (Tonner et al., 2002).

IGFBP-5 and PAI-1 are both present in ECM. Both proteins have been shown to bind to one another through the 201-218 region of C-terminus of IGFBP-5 and to influence IGF-mediated

cell responses (Nam et al., 1997). We were interested in examining whether IGFBP-5 is also involved (in an IGF-independent fashion) in the regulation of tissue remodelling via a direct influence upon the plasminogen system. Our group has also identified a specific interaction of IGFBP-5 with alpha s2-casein (αs_2-casein) (Tonner et al., 2000a). This milk protein has also been shown to bind plasminogen and its activator, tPA (Heegaard et al., 1997b) and the physical apposition of these molecules suggests that IGFBP-5 may directly influence aspects of plasminogen activation. We postulated that this binding may inhibit the action of PAI-1, which would, in turn influence the activation of plasminogen and the consequent breakdown of the ECM that takes place during tissue remodelling. Therefore, the purpose of these studies was to examine the effects of IGFBP-5 as well as IGFBP-5 mutants, which are unable to bind to IGF-I or heparin, on plasmin generation in HC11 cells.

V.2 Results

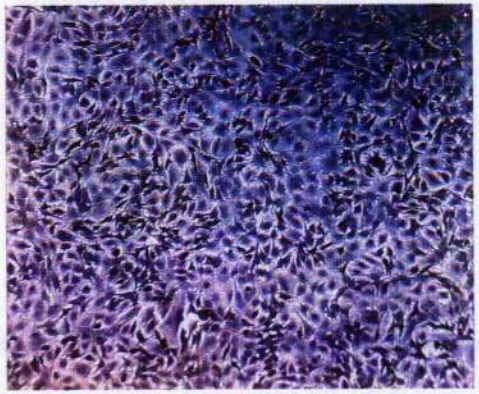
V.2.1 Effects of IGFBP-5 on plasminogen activation

Plasminogen, PAI-1, and recombinant IGFBP-5 were added to the cultures of HC-11 cells in serum-free conditions and after 24–48 hours of incubation, cultures were assayed for plasmin activity and fixed and stained with Crystal violet (See Chapter II Materials and methods).

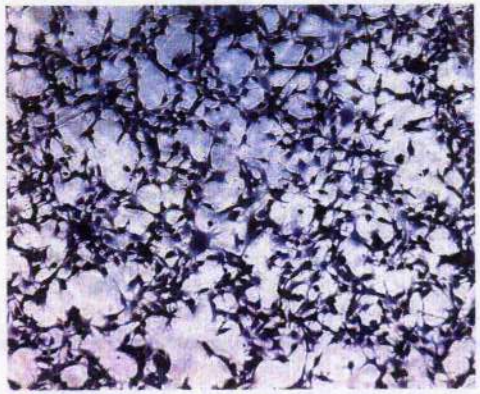
Histological results are shown in Figure V. 1. Panel A shows a confluent monolayer of HC11 cells. Addition of plasminogen alone to HC11 cells resulted in cell migration and ultimately apoptosis of the cells indicating the conversion of plasminogen (Pgen) to plasmin by plasminogen activator (PA) produced by the cells (Figure V. 1B). These plasmin(ogen)-induced effects were prevented by PAI-1, as the cells remained attached to their substratum (Figure V. 1C). However, the addition of IGFBP-5, to cultures containing PAI-1 and plasminogen again induced cell migration and ECM remodelling, indicative of the generation of plasmin (Figure V. 1D). In contrast, the empty vector (EV) control (eluate recovered from GST-affinity purification of crude lysates from bacteria containing empty pGEX 6P-1 protein expression vector), failed to influence the actions of PAI-1 (Figure V. 1E).

That these effects due to plasmin generation was confirmed by measuring plasmin activity (Figure V. 2). First, as a control, in the absence of plasminogen there was no plasmin activity. However if plasminogen was added into HC11 cultures, plasmin activity increased rapidly. With addition of PAI-1 to the plasminogen mixture, plasmin formation was indeed inhibited. Finally when IGFBP-5 was added to the mixture of plasminogen and PAI-1, it was able to reverse the effects of PAI-1 and resulted in increased plasmin generation, although the activity did not reach the level of plasmin activity achieved with plasminogen alone. This was, in part, because plasmin generation by IGFBP-5 needed some time to start up. After 36 hours of treatment, plasmin activity in the presence of IGFBP-5 eventually reached that of the plasminogen treatment (data not shown). Proteins expressed from the empty vector in bacteria were used as a control and failed to influence plasmin generation. In conclusion, IGFBP-5 was able to inhibit the action of PAI-1 on HC11 cells. Therefore, IGFBP-5 might be important as a co-ordinator of cell death and remodelling of ECM.

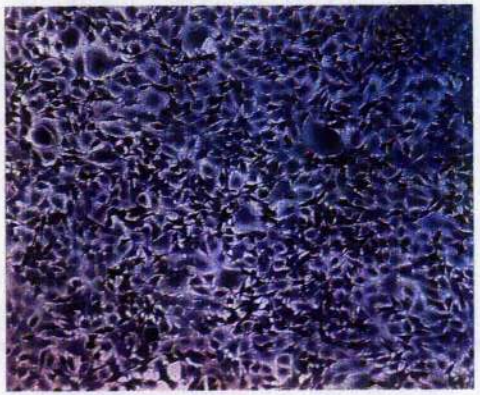
A. Control



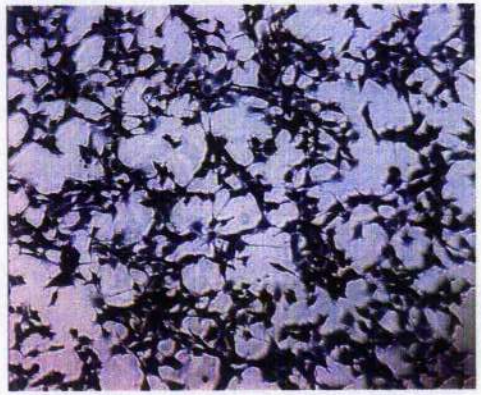
B. Pgen



C. Pgen+PAI-1



D. Pgen+ PAI-1+*wt* IGFBP5



E. Pgen + PAI-1 + pGEX-6P1 (EV)

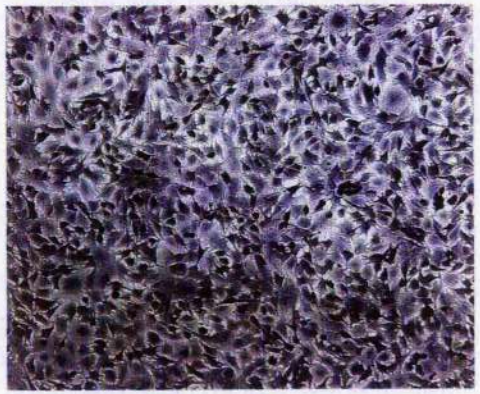


Figure V. 1 Effect of plasminogen (Pgen), PAI-1, and *wt* IGFBP-5 on HC11 cells. HC11 cells were seeded into 96 well plates and were grown to confluence in DMEM plus 2mM glutamine, 1mM sodium pyruvate, P/S (100 μg/ml), EGF(10 ng/ml) and insulin (5 μg/ml). Then the cells were washed with PBS and treated with various combinations of Pgen (4 μM), PAI-1 (800 ng/ml), and IGFBP-5 (50 μg/ml) under serum-free condition. Cells were incubated at 37 °C for 24 hours after which the culture medium was removed for determination of plasmin activity while the cells were fixed with 4 % PFA stained with crystal violet, rinsed and photographed. This experiment is representative of 15 experiments.

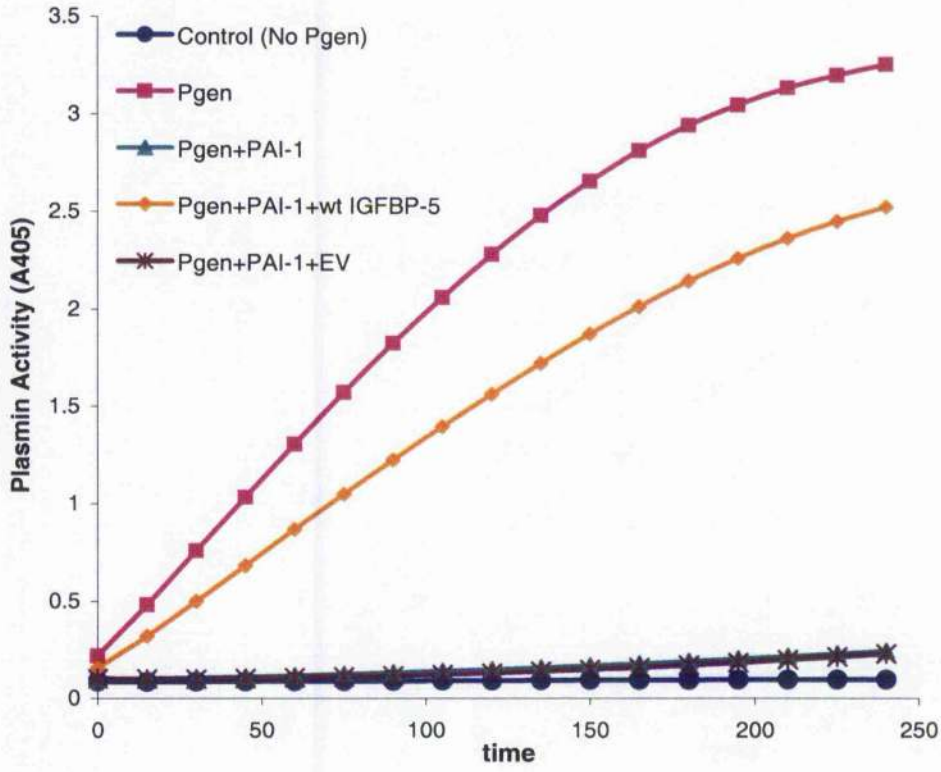
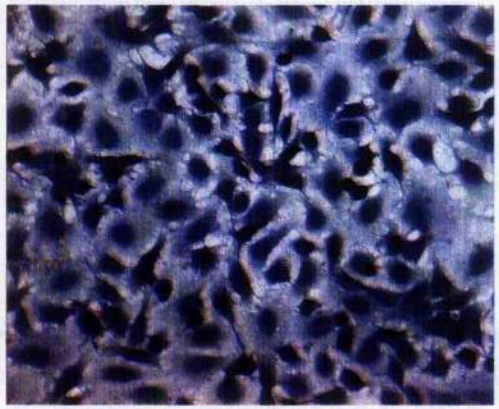


Figure V. 2 Plasmin generation in HC11 cells treated with Pgen, PAI-1 and IGFBP-5. After 24 hours culture with various combinations of Pgen (4 μM), PAI-1 (800 ng/ml), IGFBP-5 (50 μg/ml) and pGEX-6P1 (EV) (50 μg/ml) under serum-free condition, 50 μl of media was collected and mixed with 125 μl of 50 mM Tris. HCl/0.1% Tween 20 pH 7.4 and 25 μl of 6mM VLL-pNA. The reaction mixture was dispensed into 96 wells multiplate and reading was taken at A405 at 15 minutes interval over 4 hours. A representative experiment is shown. Addition of plasminogen led to rapid generation of plasmin. Addition of PAI-1 with plasminogen completely prevented this. Addition of plasminogen, PAI-1 and IGFBP-5 led to increased plasmin generation. Addition of PAI-1 plus proteins from the empty vector control failed to generate plasmin. Finally, there was no endogenous generation of plasmin in the absence of exogenous plasminogen. This experiment was performed on at least 5 occasions for each treatment.

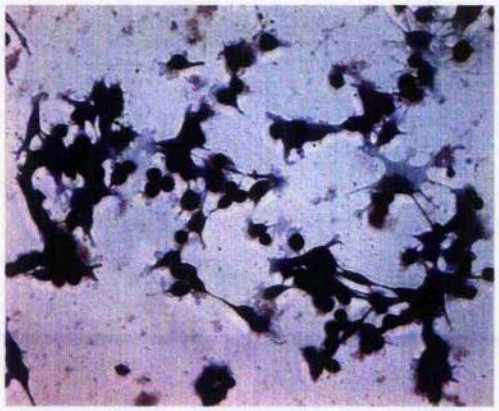
V.2.2 Effects of IGFBP-5 mutants on plasmin activation

This study was performed to compare the effects of wild-type (*wt*) IGFBP-5 and a mutant (*mt*) IGFBP-5 (non IGF-I binder, N-Term) and another that had been shown to bind poorly to heparin (Hep-). A mutant IGFBP-5 which had greatly reduced affinity for IGF-I (N-Term) was as effective as *wt* IGFBP-5 protein in terms of cell remodelling (Figure V. 3E),. A mutant HEP-, which had a greatly reduced affinity for heparin, showed only a small decrease in potency when compared *wt* IGFBP-5. Similar observations were made for C-term E and C-term F, two additional non-heparin binding mutants (results not shown).These findings were mirrored by changes in plasmin generation in the medium (Figure V. 4). This result indicated that the effect of IGFBP-5 on PAI-1 action is completely unrelated to its ability to bind to IGFs (IGF-independent function of IGFBP-5) or heparin. Biosensor analysis demonstrated that wt IGFBP-5 bound to PAI-1 with a Kd of approximately 100 nM (Figure V. 5a). However, the non-heparin binding mutant, C-term E, had a greatly reduced affinity for PAI-1 (Figure. 5b) whereas N-term had a similar affinity to *wt* IGFBP-5 (Figure 5c). Given that C-term E had little affinity for PAI-1 it seemed unlikely that it could antagonise the effects of PAI-1 by a direct molecular interaction. We therefore examined these effects of IGFBP-5 on tPA, uPA and PAI-1 activities in cell-free studies (Biosensor analysis was performed by Dr. James Beatties and submitted in Sorrell et al.).

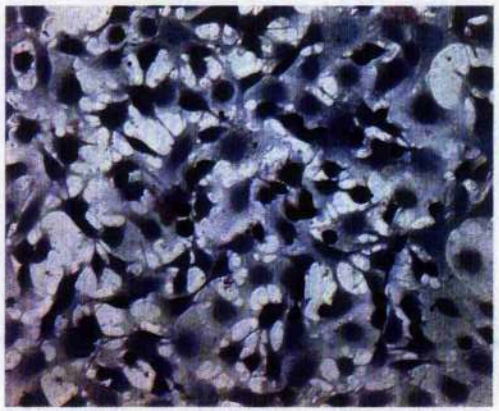
A. Control



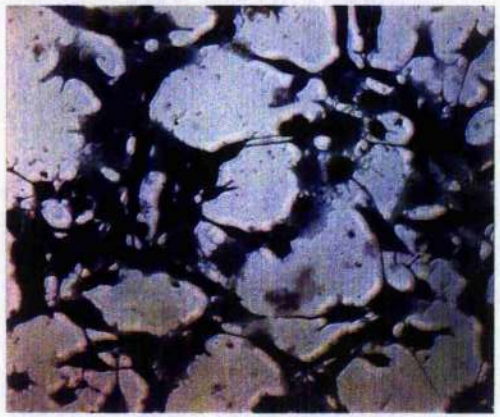
B. Pgen



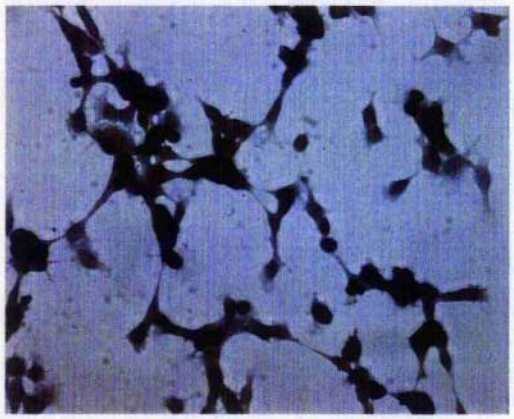
C. Pgen+PAI-1



D. Pgen+PAI-1+ *wt* IGFBP-5



E. Pgen+PAI-1+N-term *mt* IGFBP-5



F. Pgen+PAI-1+Hep- *mt* IGFBP-5

Figure V. 3 Effect of *wt* IGFBP-5, *mt* IGFBP-5 (N-Term and HEP-) on HC11 cells treated with Pgen, and PAI-I. wt IGFBP-5, an IGFBP-5 mutant with greatly reduced affinity for IGF-I (N-term) and an IGFBP-5 mutant with greatly reduced affinity for heparin (C-term E) were all capable of preventing the inhibitory effect of PAI-I on cell death. HC11 cells were seeded into 96 well plates and grown to confluence in DMEM. The HC11 cells were then washed with PBS and treated with various combinations of Pgen (4 uM), PAI-1 (800 ng/ml), and IGFBP-5 (50 µg/ml) or N-Term *mt* IGFBP-5 (50 µg/ml), Hep- *mt* IGFBP-5 (50 µg/ml) under serum-free condition. Cells were incubated at 37 °C for 24 hours. After 24 hours, the culture medium was removed for plasmin activity assay while the cells were washed with PBS to remove traces of medium. 100ul of 4% PFA was added to wells for a minimum of 20 minutes to fix cells. Wells were washed with PBS twice before addition of Crystal violet for a minimum of 5 min. Cells were then washed with ddH_2O until washes were clear. Photographs were taken with an inverted microscope.

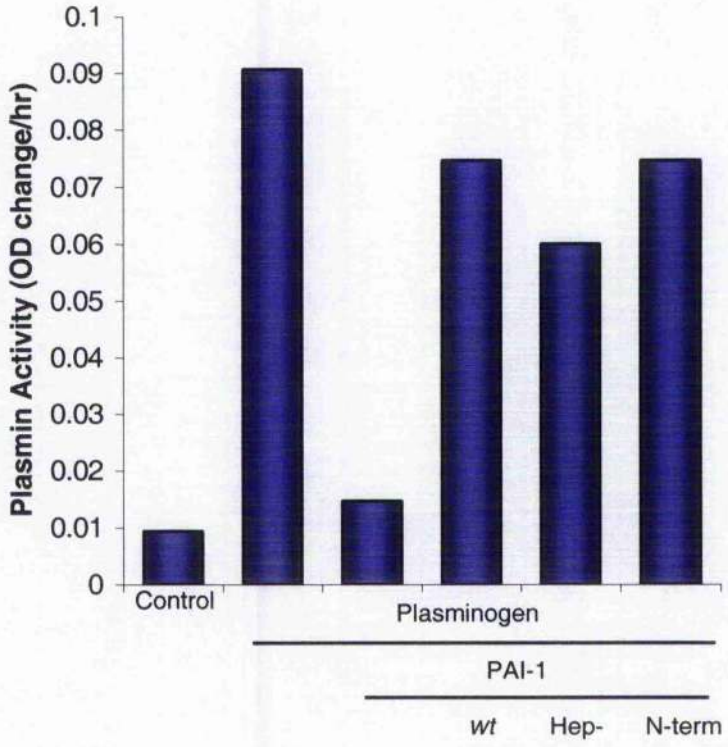


Figure V. 4 Plasmin generation in HC11 cells treated with mixture of Pgen, PAI-1 and either *wt* or *mt* IGFBP-5. After 24 hours culture with various combination of Pgen (4 uM), PAI-1 (800 ng/ml), and IGFBP-5 (50 μg/ml) or N-term *mt* IGFBP-5 (50 μg/ml), Hep- *mt* IGFBP-5 (50 μg/ml) under serum-free condition, 50ul of media was collected and mixed with 125ul of Tris HCl/0.1% Tween 20 pH 7.4 and 25ul of 6mM VLL. The reaction mixture was dispensed into 96 wells multiplate and reading was taken at A405 at 15 minutes interval over 4 hours. Plasmin activity was measured by OD change per hour. Plasmin generation was inhibited by PAI-1 but increased if *wt* IGFBP-5, N-term *mt* IGFBP-5 or HEP- *mt* IGFBP-5 were also added to the medium. Results represent mean plasmin activities from 3 culture wells. The result is representative of studies which were conducted on 4 occasions.

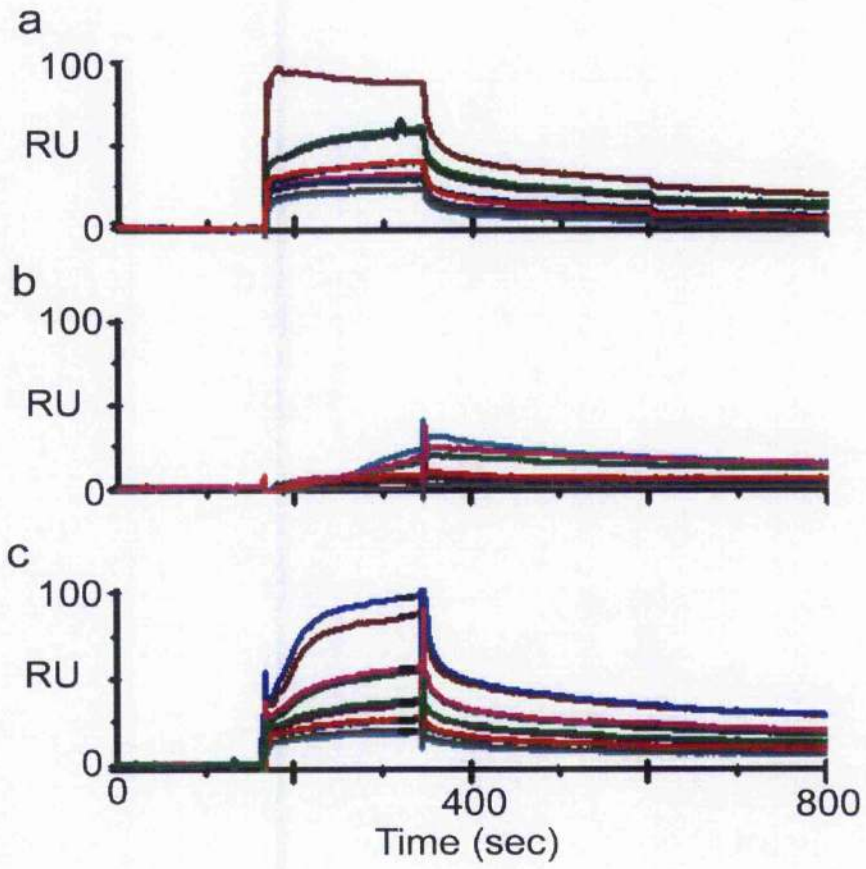


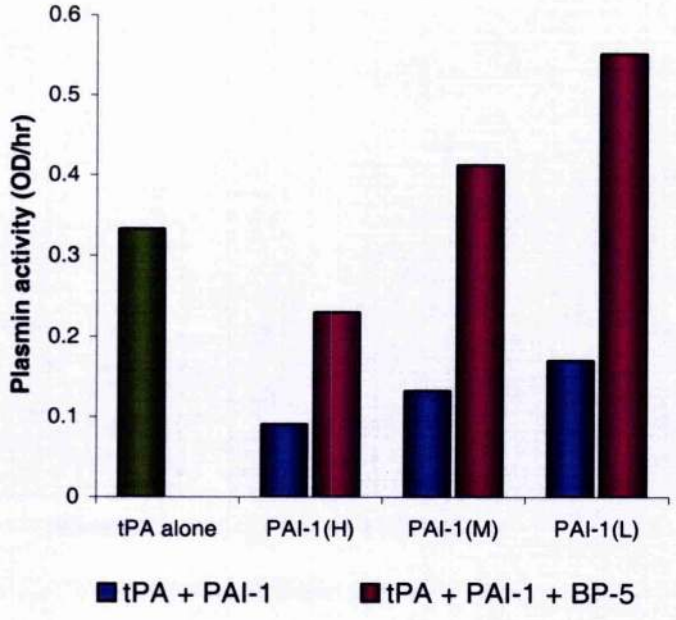
Figure V. 5 Biosensor analysis of interactions of PAI-1 with IGFBP-5. Individual sensorgrams for *wt* IGFBP-5 (a), C-term E (b) and N-Term (c) binding to human PAI-1 ligand, which was covalently captured, by amine coupling chemistry, to the carboxy-methyl surface of a CM5 biosensor chip. RU = resonance units.

V.2.3 Effects of IGFBP-5 on tPA/uPA

There are two different plasminogen activators, the urokinase-type (uPA) and tissue-type (tPA), which enhance the conversion of plasminogen to plasmin. We have identified a specific interaction of IGFBP-5 with alpha s2-casein (αs_2-casein) (Tonner et al., 2000b). This milk protein has also been shown to bind plasminogen and one of its activators, tPA (Heegaard et al., 1997a). IGFBP-5 was also present in the micellar fraction of milk which contains most of the casein. This study was carried out to investigate the relationship between IGFBP-5 and uPA or tPA. HC11 cells are known to express PA, thus we used a cell-free system in which we could control the amount PA. Initially, we examined the effect of tPA and uPA alone on plasmin generation.

As shown in Figure V. 6A, tPA and uPA alone acted upon plasminogen to generate plasmin. If PAI-1 was added to tPA/uPA and plasminogen mixture, plasmin production was inhibited in a dose dependent manner. When IGFBP-5 was added into the tPA/PAI-1 mixture, we showed that IGFBP-5 was able to inhibit the effect of PAI-1 in a dose-dependent fashion. However, at a lower dose of PAI-1, IGFFBP-5 not only inhibited the effect of PAI-I but actually increased plasmin production to levels even greater than with tPA alone. Furthermore, when IGFBP-5 was incubated with uPA plus PAI-1, IGFBP-5 failed to inhibit the action of PAI-1 (Figure V. 6B). These results suggested that IGFBP-5 was acting, not by inhibiting PAI-1, but by enhancing the activity of tPA but not uPA. We therefore tested the effect of IGFBP-5 on uPA or tPA plus plasminogen in the absence of PAI-1. This confirmed our conclusion, since IGFBP-5 enhanced the effect of tPA on plasminogen in a dose-dependent fashion (Figure V. 7A) whereas plasmin generation by uPA was not enhanced by IGFBP-5 (Figure V. 7B). BSA was used as a control and showed no ability to enhance tPA or uPA activity.

A.


Plasmin activity (OD/hr)
0.6
0.5
0.4
0.3
0.2
0.1
0
tPA alone
PAI-1(H)
PAI-1(M)
PAI-1(L)
tPA + PAI-1
tPA + PAI-1 + BP-5

B.

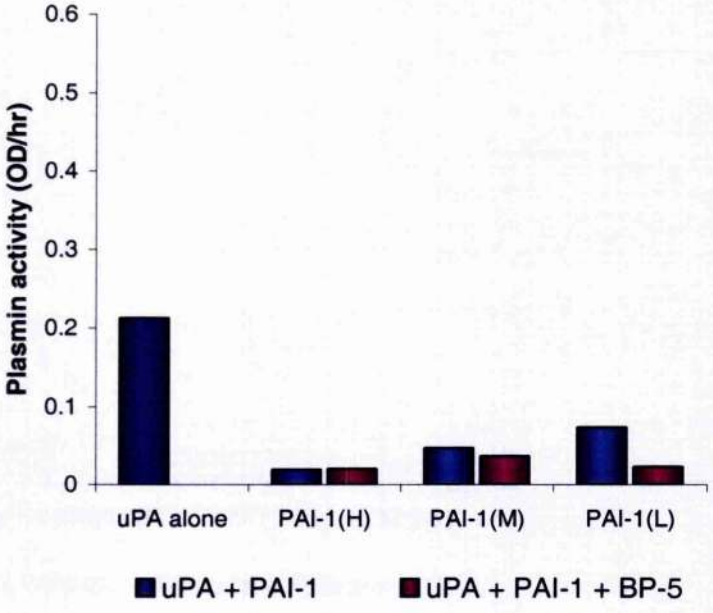
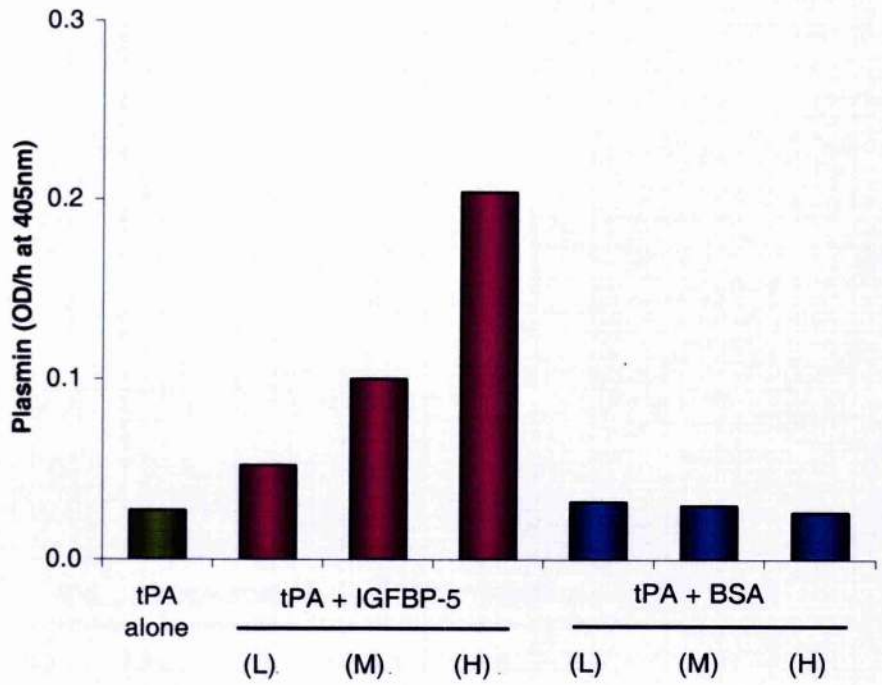

Plasmin activity (OD/hr)
0.6
0.5
0.4
0.3
0.2
0.1
0
uPA alone
PAI-1(H)
PAI-1(M)
PAI-1(L)
uPA + PAI-1
uPA + PAI-1 + BP-5

Figure V. 6 Effect of tPA (A)/uPA (B) and IGFBP-5 on plasmin generation.

A. tPA 200 ng/ml was incubated with 10 µl of 6mM VLL-pNA, 10 µl of 2mM Plasminogen, PAI-1(High): 1000 ng/ml, PAI-1(Medium): 500 ng/ml, PAI-1(Low): 250 ng/ml, with or without IGFBP-5 (25 µg/ml) in Tris HCl, 0.1% Tween 20, pH 7.4 in a total volume of 140 µl. Plasmin Activity was measured at A405 at 15 minutes interval for 4 hours. Then plasmin activity was assessed by OD change per hour. Results are means of triplicate wells and are representative of results obtained in 3 independent experiments.

B. uPA 115 ng/ml was incubated with 10 µl of 6mM VLL-pNA, 10 µl of 2mM Plasminogen, PAI-1(H): 1000 ng/ml, PAI-1(M): 500 ng/ml, PAI-1(L): 250 ng/ml, with or without IGFBP-5 (25 µg/ml) in Tris HCl, 0.1% Tween 20, pH 7.4 in a total volume of 140 µl. Plasmin Activity was measured at A405 at 15 minutes interval for 4 hours. Then plasmin activity was assessed by OD change per hour. Results are means of triplicate wells and are representative of results obtained in 3 independent experiments.

A.


0.3
0.2
0.1
0.0
Plasmin (OD/h at 405nm)
tPA alone
tPA + IGFBP-5
(L)
(M)
(H)
tPA + BSA
(L)
(M)
(H)

B.

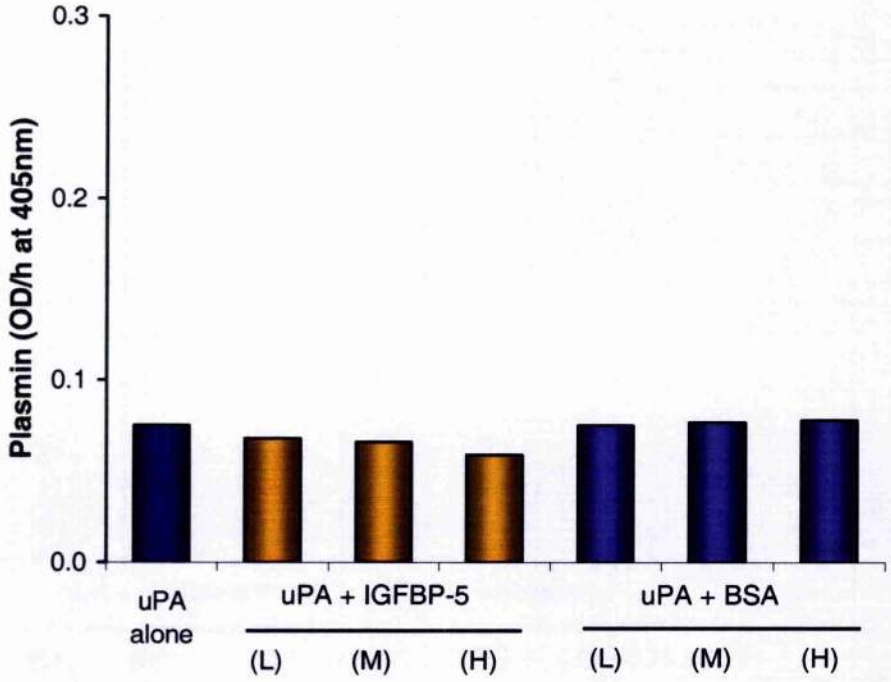

0.3
0.2
0.1
0.0
Plasmin (OD/h at 405nm)
uPA alone
uPA + IGFBP-5
(L)
(M)
(H)
uPA + BSA
(L)
(M)
(H)

Figure V. 7 Effects of IGFBP-5 and tPA(A)/uPA(B) on plasmin generation.

A. tPA 50 ng/ml was incubated with 10 µl of 6mM VLL, 10 µl of 2 mM Plasminogen, without or with IGFBP-5(L): 12.5 µg/ml, IGFBP-5(M) 25 µg/ml, IGFBP-5(H) 50 µg/ml or BSA(control) at the same concentrations, in Tris HCl 0.1% Tween 20, pH 7.4 in a total volume of 140 ul. Plasmin activity was determined as OD change per hour. Plasmin Activity was measured at A405 at 15 minutes interval for 4 hours. Then plasmin activity was assessed by OD change per hour.

B. uPA 138 ng/ml was incubated with 10 µl of 6 mM VLL, 10 µl of 2 mM Plasminogen, without or with IGFBP-5(L): 12.5 µg/ml, IGFBP-5(M) 25 µg/ml, IGFBP-5(H) 50 µg/ml or BSA(control) at the same concentrations, in Tris HCl 0.1% Tween 20, pH 7.4 in a total volume of 140 µl. Plasmin activity was determined as OD change per hour, Plasmin Activity was measured at A405 at 15 minutes interval for 4 hours. Then plasmin activity was assessed by OD change per hour.

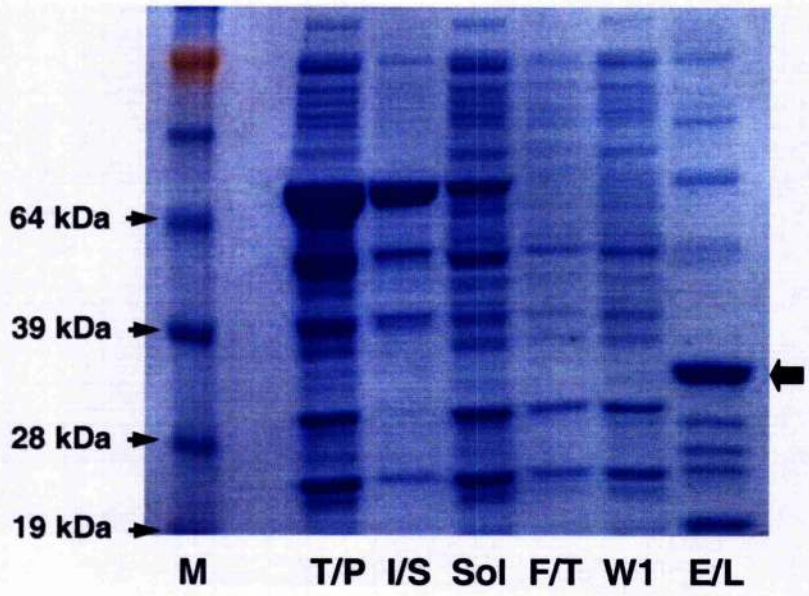
V.2.4 Effects of purified IGFBP-5 on plasmin generation

Although the IGFBP-5 proteins purified by glutathione-sepharose were relatively pure, we did note, in some preparations, the presence of impurities (Figure V. 8A). Western blotting with an anti-IGFBP-5 polyclonal antibody revealed many of the smaller molecular weight impurities to be fragments of IGFBP-5 (data not shown). However, concerned that these impurities might also include protease contamination (either of bacterial origin or the PreScission protease used to cleave off the GST tag) we further purified IGFBP-5, on an IGF-affinity column (Figure V.8B). This produced IGFBP-5 devoid of fragments, as well as a mixture of lower molecular weight proteins generated from the flow-through (unbound fraction) from the IGF-affinity column. These preparations were investigated for their ability to enhance plasmin generation. Serum-starved HC11 cells were incubated with IGFBP-5 purified from the glutathione column, IGFBP-5 (IGF-affinity purified) or contaminating proteins not bound by the IGF column, together with PAI-1and plasminogen for 24-48 hours. As shown in Figure V. 9, plasmin activation by glutathione-purified IGFBP-5 once again resulted in cell death. IGFBP-5 purified by IGF-affinity chromatography also induced cell death although the effect was less dramatic. However, fragments from the IGF-affinity purification procedure also had considerable activity (Figure V. 9). This was also evident from the determination of plasmin activation, where quantitative analyses were possible. These suggested that compared with the glutathione-purified material, purified IGFBP-5 had about 25 % of the activity and the fragments about 50 % of the activity. The fragments thus possessed about twice the activity of affinity-purified IGFBP-5 on a weight basis although this would clearly be less on a molar basis (Figure V. 10).

To further assess potential artefacts, we first examined the possibility that PreScission Protease was contaminating the glutathione-purified preparation and cleaving the substrate instead of plasmin. However, two experiments ruled out this possibility. Firstly, the activity of PreScission Protease was extremely low in the plasmin assay. Thus, even if the preparation had been contaminated with 100 % of the PreScission protease used this would still only have explained 10-20 % of the activity of the glutathione-purified IGFBP-5 (Figure V. 11). Secondly, the glutathione-purified IGFBP-5 preparation was passed through the glutathione-sepharose column for a second

time to remove any residual PreScission protease. This resulted in a 20 % loss of plasmin generation activity but this was also evident in samples which were incubated with unmodified sepharose or were simply diluted and incubated for the same periods time, presumably reflecting non-specific losses of IGFBP-5. Thus the increased plasmin activity could not be explained by contamination with PreScission Protease. Likewise, the low activity of empty vector preparations indicated that this was not some form of bacterial protease contaminating the preparations (Figure IV. 12).

A. GST-affinity chromatography



B. IGF-affinity

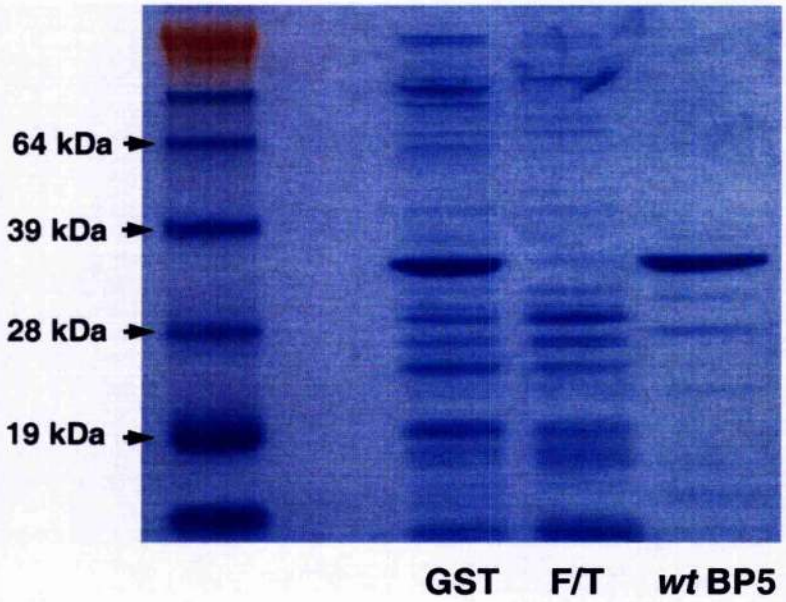
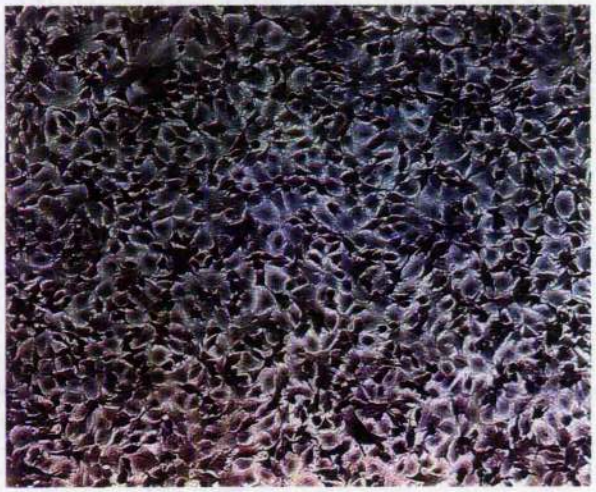
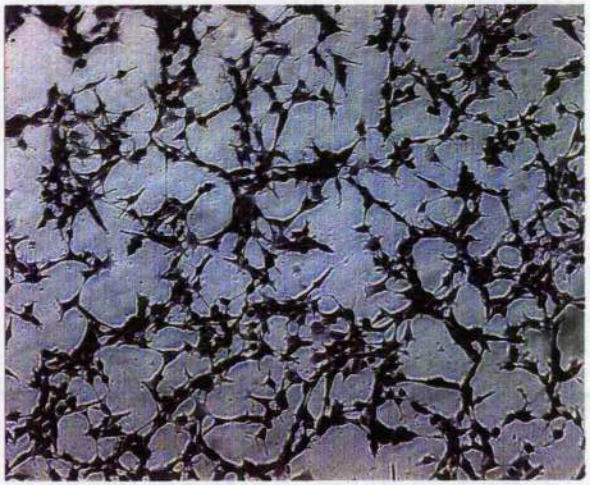


Figure V. 8: A. Purification of IGFBP-5 expressed as a GST-fusion protein in E. coli. Bacterial cells were harvested and lysed (original). Insoluble material (I/S) was removed by centrifugation, then the supernatant (Sol) was filtered through a 0.45-μm pore size membrane and incubated overnight at 4 °C with 1 ml (packed volume) washed glutathione-Sepharose. The suspension was decanted into a disposable plastic column and unbound material allowed to flow through (F/T), then the glutathione-Sepharose was washed with 10 ml PBS (W1). The column was sealed, and the glutathione-Sepharose was resuspended in 2 ml cleavage buffer containing 160 U PreScission protease. After 4 hour at room temperature with hourly resuspension, the column was reopened, and the cleaved IGFBP-5 was recovered in the eluate (E/L). Samples were resolved on NuPAGE 10 % Bis.Tris gel. The eluted material (E/L) contained a major band at the appropriate molecular wieght for intact IGFBP-5 (indicated by arrowhead) along with smaller molecular wt fragments and some high molecular wt contaminants. **B. IGF-affinity purification.** Samples were resolved on NuPAGE 12 % Bis.Tris gel. Subsequent purification of this material (GST) on an IGF-I affinity column produced an unbound fraction (F/T) containing contaminating proteins and a bound fraction which was eluted (*wt*BP5) and contained essentially pure intact IGFBP-5.

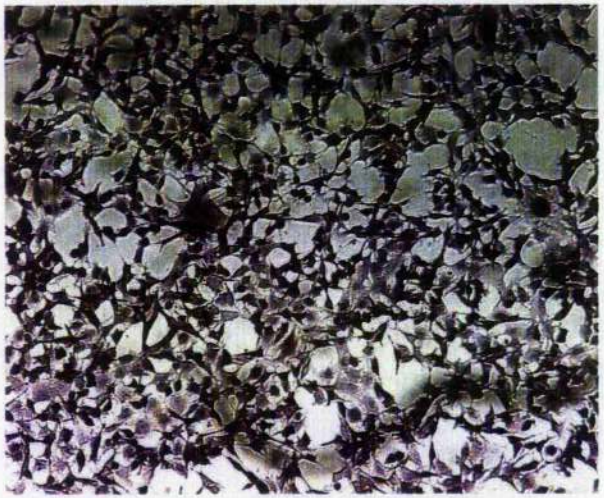
A. Serum free Control



B. Pgen + PAI-I + GST-affinity purified



C. Pgen + PAI-I + IGF-affinity purified



D. Pgen + PAI-I + Fragments

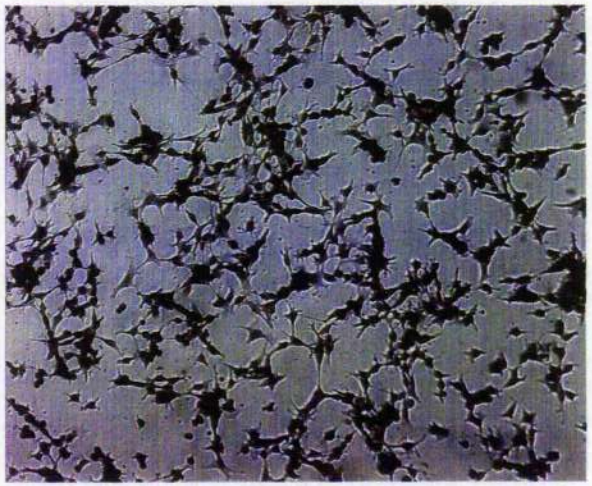


Figure V. 9 Comparison of the effects of IGFBP-5 and fragments. HC11 cells were seeded into 96 well plates and grown to confluence in DMEM plus 2 mM glutamine, 1mM sodium pyruvate, P/S (100 µg/ml), EGF (10 ng/ml) and insulin (5 µg/ml). The HC11 cells were then washed with PBS and treated with various combinations of Pgen (4 µM) and PAI-1 (800 ng/ml), together with IGFBP-5, IGF-affinity purified IGFBP-5, or fragments (all at 50 µg/ml) under serum-free conditions. Cells were incubated at 37 °C for 24 hours. After 24 hours, the culture medium was removed for plasmin activity assay while the cells were washed with PBS to remove traces of medium. 100 µl of 4% PFA was added to the wells for a minimum of 20 min to fix cells. Cells were washed with PBS twice, after which Crystal violet was added for at least 5 min. Cells were washed wells with ddH2O until washes were clear. Photographs were taken with an inverted microscope.

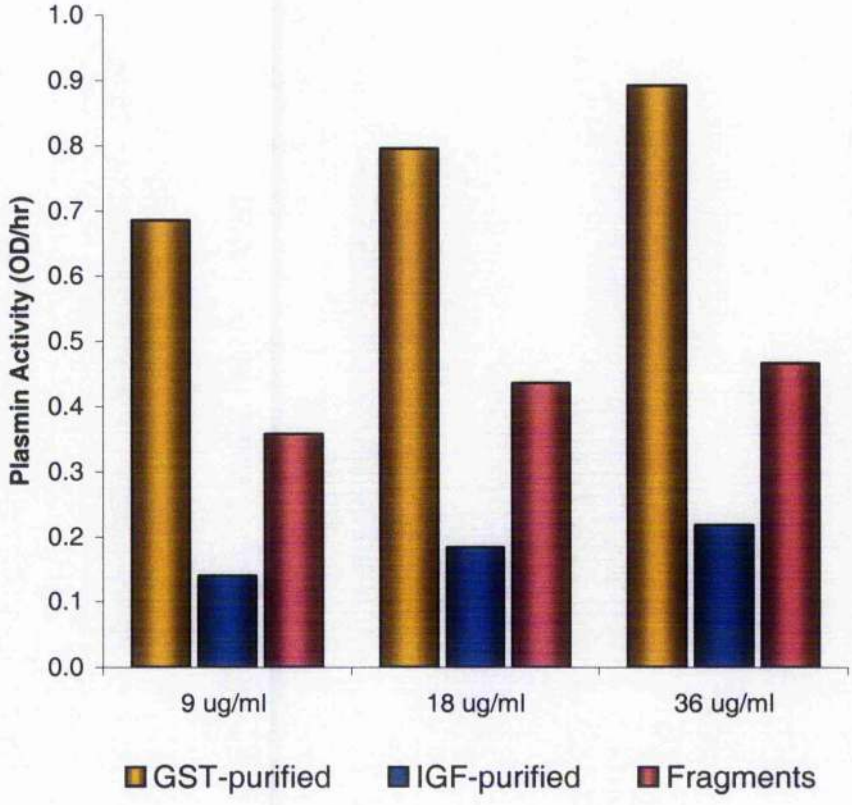


Figure V. 10 Enhancement of tPA activity by IGFBP-5 purified by glutathione sepharose, IGFBP-5 further purified by IGF-affinity chromatography or low molecular weight contaminants. Results are means of triplicate wells.

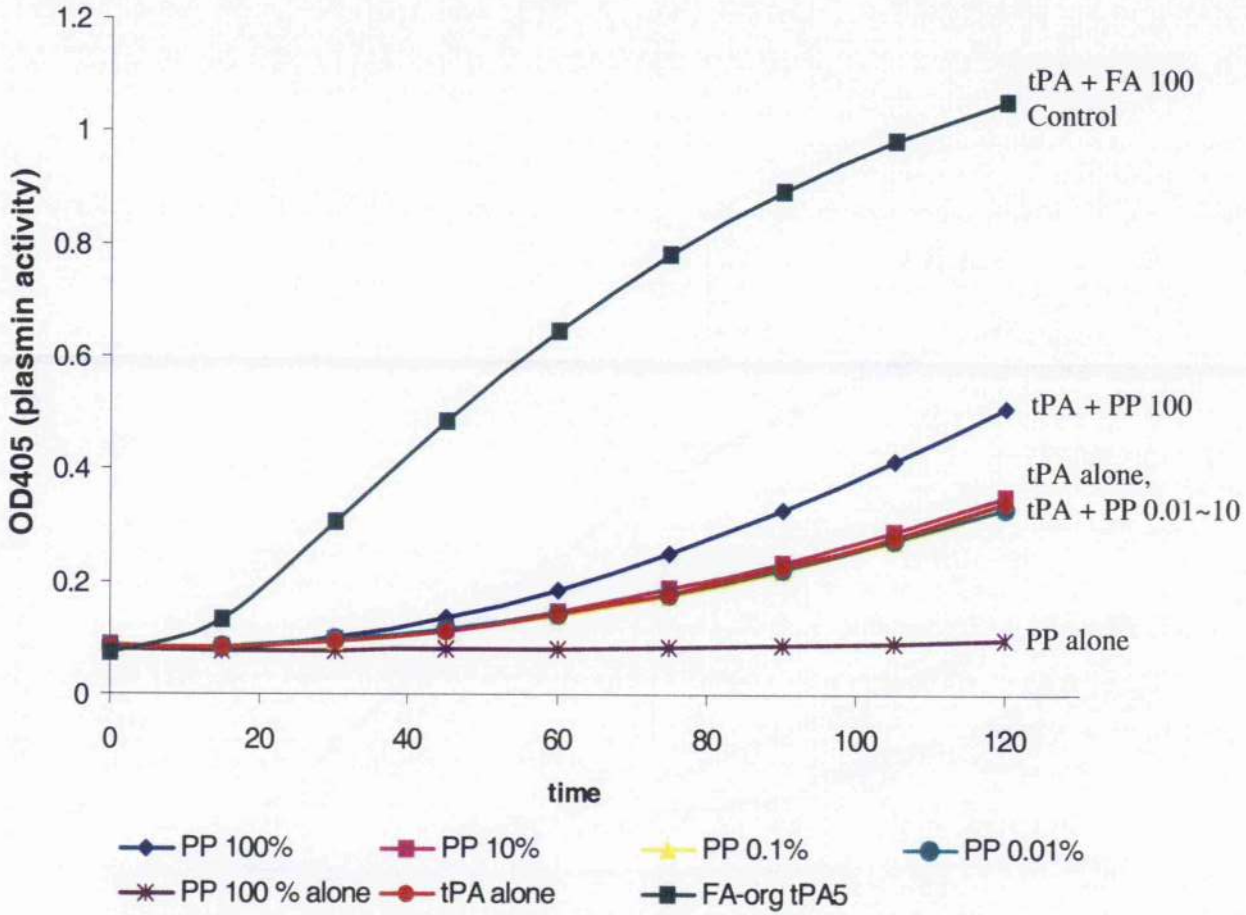

1.2
1
0.8
0.6
0.4
0.2
0
OD405 (plasmin activity)
0
20
40
60
80
100
120
time
tPA + FA 100
Control
tPA + PP 100
tPA alone,
tPA + PP 0.01~10
PP alone
PP 100%
PP 10%
PP 0.1%
PP 0.01%
PP 100 % alone
tPA alone
FA-org tPA5

Figure V. 11 Activation of tPA by IGFBP-5 is not due to contamination with Precission protease. The activity of tPA alone is shown in red circles. Precission protease (PP) had no intrinsic activity against the substrate VLL-pNA (purple crosses). Furthermore, it had no ability to activate tPA when added with tPA at various concentrations equivalent to up to 10% contamination of the IGFBP-5 preparation (values superimposable with tPA alone). Even assuming a 100% contamination of the IGFBP-5 preparation with precission protease, this combination of tPA plus precission protease (blue diamonds) could only provide 10% of the activation ability when C-term F IGFBP-5 was incubated with tPA (green squares).

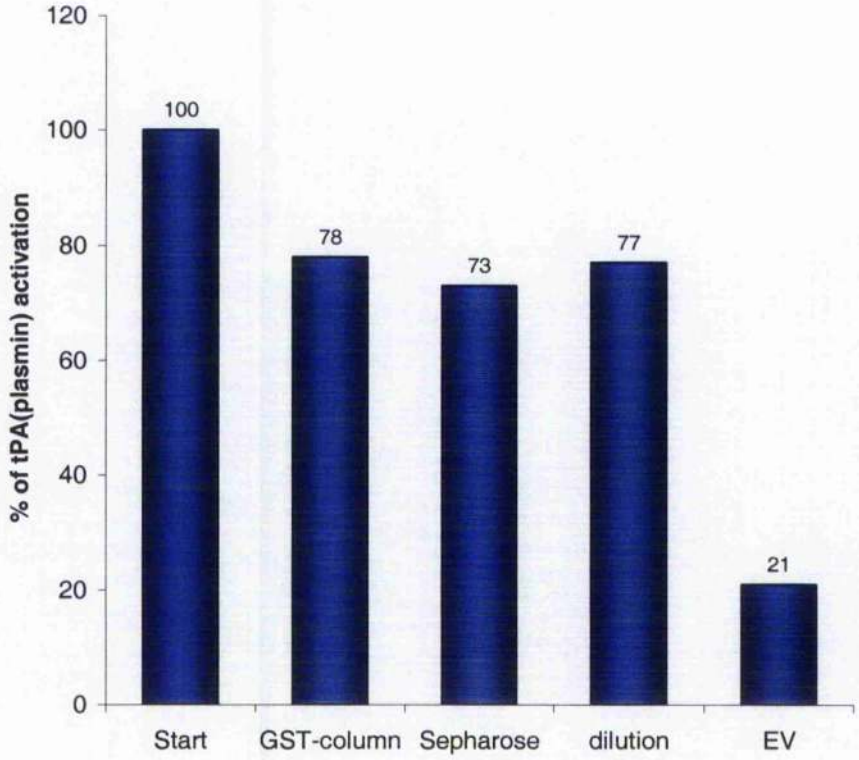


Figure V. 12 tPA activation potency of C-term F IGFBP-5/Empty vector (EV). The glutathione-purified IGFBP-5 C-term F mt IGFBP-5, non heparin binding mutant, (start) preparation was passed through the glutathione-sepharose column (GST-column) for a second time to remove any residual PreScission protease or incubated with unmodified sepharose (Sepharose) or simply diluted with buffer (dilution) for the same periods of time. Relative tPA activtion was measured by plasmin activation against start material.

V.2.5 Purification of fragments

Plasmin activation was compared between GST-purified, IGF affinity purified and *wt* IGFBP-5 purified by Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) (Figure V. 13). Plasmin activation from all IGFBP-5 preparations increased in a dose-dependent manner. GST-purified IGFBP-5 had greater plasmin activity than either IGF-purified or RP-HPLC purified intact IGFBP-5. However, IGF-affinity purified and RP-HPLC purified IGFBP-5 had almost identical plasmin activity. Intact IGFBP-5 from either IGF-affinity purification or RP-HPLC purification had approximately 50% of the activity of GST-purified IGFBP-5 and we therefore concluded that the rest of activity was present in the fragments of IGFBP-5.

Figure V. 14A shows the RP-HPLC elution profiles of the GST-purified recombinant C-term E IGFBP-5 mutant. Most of the intact IGFBP-5 was present in fractions 35-37 (Figure V. 14B). We then investigated the plasmin activation on each fraction. Figure V. 15 shows the plasmin activity of each fraction. Fraction 43 had the greatest plasmin activity among the fractions, even greater than GST-purified IGFBP-5, whereas all other fractions had less activity than GST-purified material. Fraction 43 had twice as much activity as GST-purified IGFBP-5, whereas intact IGFBP-5 had half the activity of GST-purified IGFBP-5. SDS-PAGE analysis showed the candidate proteins, potentially responsible for enhancing tPA activity (Figure V.16). However, fraction 43 had at least 7 distinct bands.

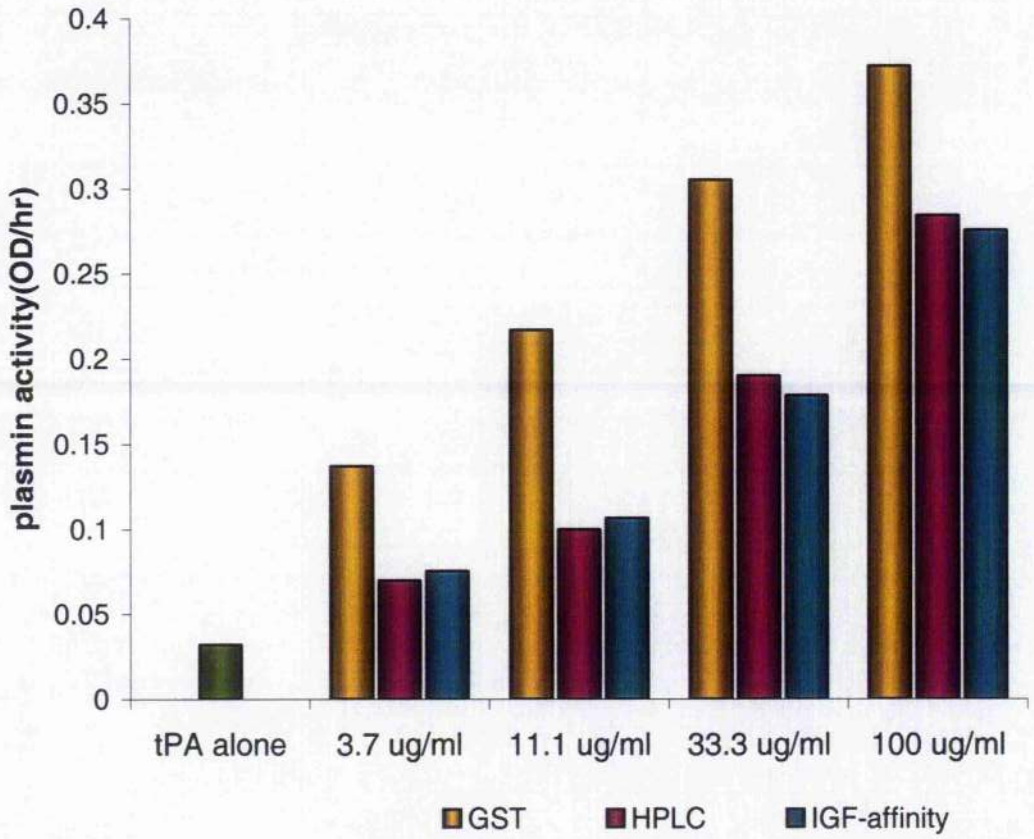


Figure V. 13 Enhancement of tPA activity by *wt* IGFBP-5 purified by glutathione-sepharose or further purified, either by IGF-affinity chromatography or RP-HPLC. Results are means of triplicate wells.

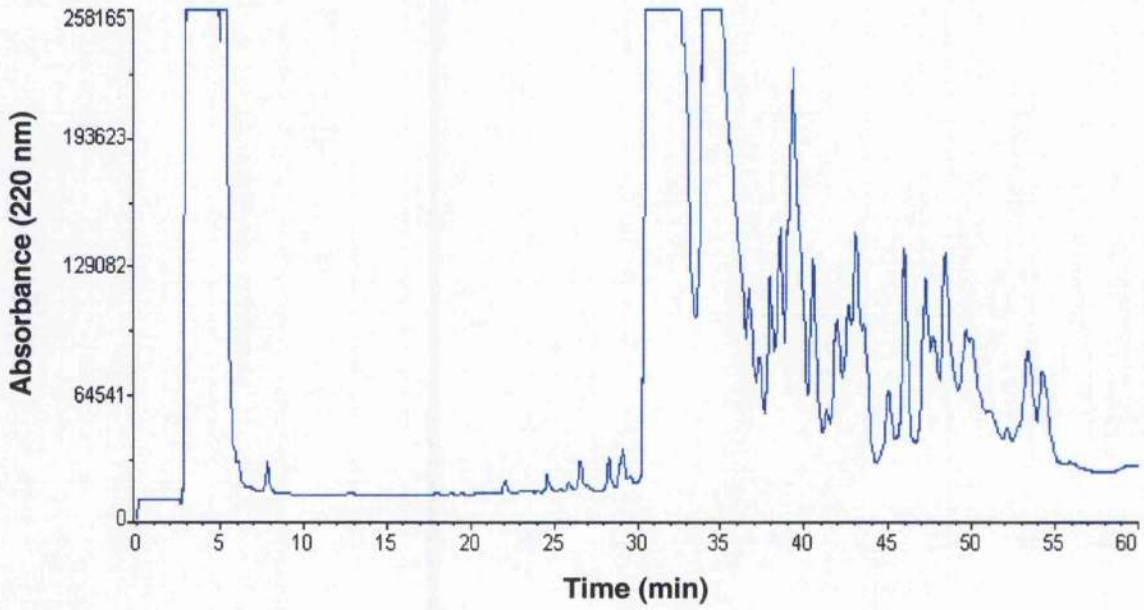


Figure V. 14A RP-HPLC chromatogram of GST-purified C-term E IGFBP-5 mutant.

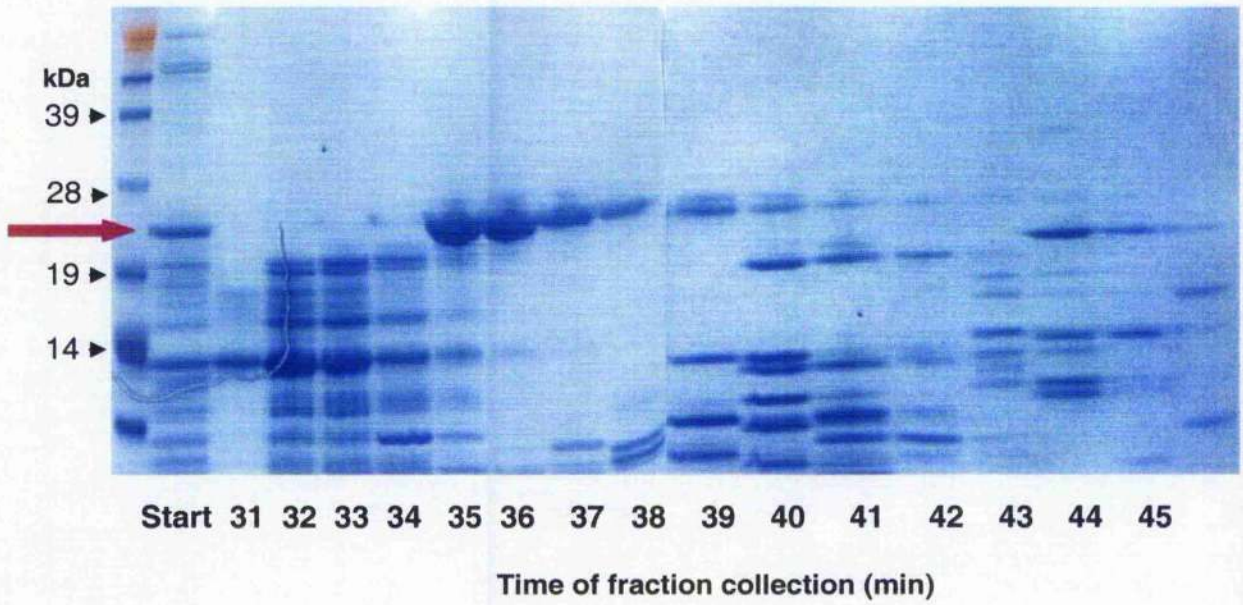


Figure V. 14B SDS-PAGE analysis of RP-HPLC fractions. Intact C-term E IGFBP-5 is indicated with an arrow.

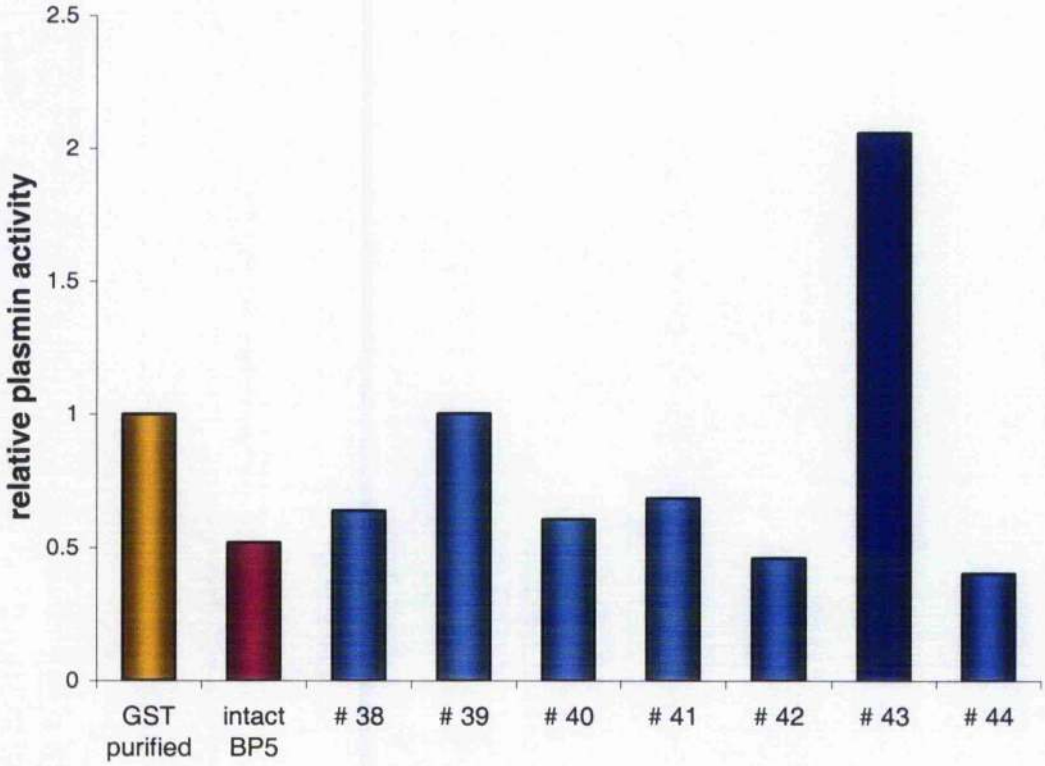


Figure V. 15 Plasmin activity in various fractions derived from RP-HPLC. Plasmin activity from each fraction of RP-HPLC procedure. Relative activity was measured against GST-affinity purified C-term E IGFBP-5. Intact IGFBP-5 was collected from fraction 35 to 37. No plasmin activity was present in the other fractions (31-35, 45, 46).

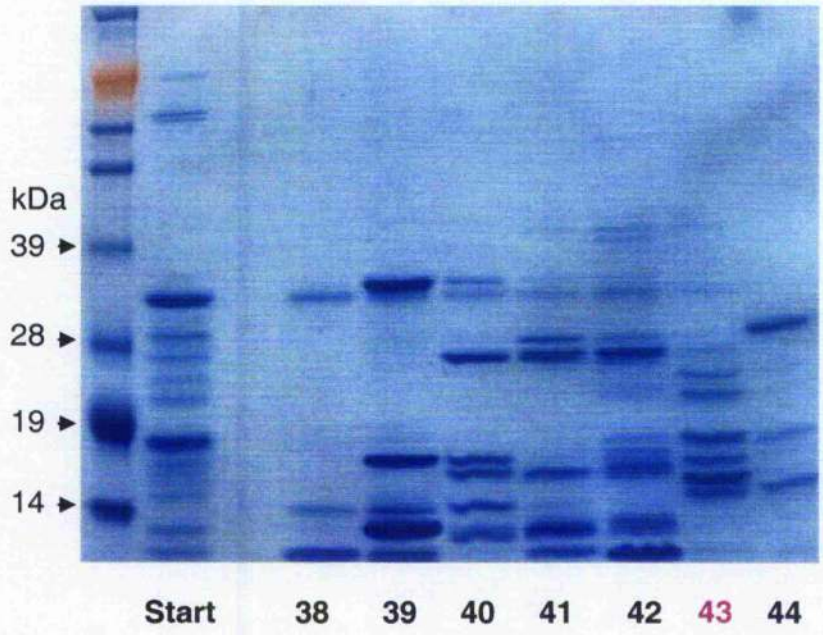


Figure V. 16 Further SDS-PAGE analysis of RP-HPLC-purified C-term E IGFBP-5 mutant. Samples were resolved on NuPAGE 12 % Bis.Tris gel and coomassie blue stained. Each lane has equal quantity of protein. (1 μg/lane).

V.3 Discussion

Our group has previously shown a large increase in the production of IGFBP-5 by the mammary gland during involution and proposed that IGFBP-5 exerts an apoptotic effect by abolishing the survival effects of IGF-I (Tonner et al., 1997). The theory that IGFBP-5 is involved in apoptosis of the mammary gland during involution has been supported by more recent studies, involving transgenic animals expressing IGFBP-5 in the mammary gland (Tonner et al., 2002) as well as exogenous treatment with IGFBP-5 both *in vivo* (Allan et al., 2002) and *in vitro* (Marshman et al., 2003). The IGFBP-5 over-expressing transgenic mouse model also exhibited increased generation of plasmin in the mammary gland and, since a specific interaction between IGFBP-5 and PAI-1 had been described (Nam et al., 1997) we examined whether IGFBP-5 could activate the plasmin system by binding to and influencing the actions of PAI-I. Utilising an epithelial cell line, HC11, we were able to show plasmin generation directly by determination of its activity in the conditioned medium. We also demonstrated that PAI-I could effectively inhibit plasmin generation in this system and that IGFBP-5 could prevent these effects of PAI-1. This clearly suggested that the physical interaction of IGFBP-5 with PAI-1 described by (Nam et al., 1997), served to inhibit the actions of PAI-1.

Furthermore we showed that an IGFBP-5 mutant, which was unable to bind to IGF-I was also able to overcome the effects of PAI-1. Therefore, this effect was IGF-independent. In addition, mutants which had poor binding to heparin showed inhibitory effects on PAI-I. This was somewhat surprising given that PAI-1 has been described to interact with IGFBP-5 via its heparin-binding domain (amino acids 201-218) (Nam et al., 1997). Our group has examined IGFBP-5 interactions with PAI-1 using Biosensor analysis and showed that IGFBP-5 did bind to PAI-1 whereas a mutant (C-term E) which bound heparin very weakly, showed greatly compromised binding to PAI-1 (See Figure V. 5), supporting the earlier finding of Clemmons and co-workers (Nam et al., 1997). It was thus puzzling that a mutant with limited ability to bind to PAI-1 could nevertheless prevent its effects on plasmin generation. However we were able to show that IGFBP-5 activated plasminogen independently of PAI-1, presumably specifically enhancing the activity of tPA directly, since it was unable to influence the effects of uPA and had no intrinsic plasminogen-cleaving activity itself.

This also implies that the interaction of IGFBP-5 with tPA lies outwith the basic residues of the heparin binding domain of IGFBP-5 and presumably outwith the major IGF-binding residues (G203, Q209) present in the N-terminus of IGFBP-5, since N-term was able to enhance tPA activity to a similar extent as *wt* IGFBP-5. However direct binding of tPA and IGFBP-5 remains to be confirmed by Biosensor analysis.

Although we have excluded the possibility that these effects originated from any contamination of either PreScission Protease or bacterial protease (see Figure V. 10 and 11), fragments from the IGF-affinity purification also induced cell death. Furthermore, the fragments possessed about twice the activity of IGF-affinity purified IGFBP-5 on a weight basis. We also showed that intact IGFBP-5 from either IGF-affinity purification or RP-HPLC purification had approximately 30 % of the activity of GST-affinity purified IGFBP-5 and therefore concluded that the remainder of activity comes from fragments of IGFBP-5. These fragments were shown to be IGFBP-5 related protein, since we could identify them with IGFBP-5 western blotting, since the GST tag is attached to the N-terminus, any fragments purified on the glutathione column would be anticipated to be c-terminally truncated. Therefore we could argue that the N-terminal region of IGFBP-5 is important for plasmin activation, although the IGF-I binding site has shown to be not important for this activation. Therefore this led us to investigate the origin of the plasmin activation effect. I examined the plasmin activation of each fraction and discovered that fraction 43 had twice as much activity as GST-purified IGFBP-5, whereas intact IGFBP-5 had half the activity of GST-purified IGFBP-5. Therefore it is possible that the effect of IGFBP-5 on the plasmin system also originates from fragments in this fraction. However, SDS-PAGE analysis showed the fraction potentially responsible for enhancing tPA activity had at least 7 distinct bands (Figure IV. 15). Time did not permit me to pursue this preliminary data any further and all that can be concluded is that there appear to be fragments with similar activity to intact IGFBP-5 on a molar basis and identification of such molecules might provide a useful therapeutic agent.

Intriguingly, our group has shown that IGFBP-5 binds to casein micelles and interacts exclusively with αs2-casein on ligand blots (Tonner et al., 2000b). It has been proposed that casein micelles thus provide a matrix for plasmin generation in milk and that this serves to prevent

formation of casein (milk) clotting. Although IGFBP-5 may play a role in maintaining potency of mammary ducts, the PA content of rodent mammary gland is also correlated with involution and thus plasminogen activation is also considered to be involved in the later stages of tissue remodelling (Ossowski et al., 1979). In rodents an increase in PA production and a decrease in PAI-1 activity are correlated with the destruction of the basement membrane and loss of the secretory cells during mammary gland involution (Busso et al., 1989; Ossowski et al., 1979; Talhouk et al., 1992). Thus IGFBP-5 plays a dual role in mammary gland remodelling during the involutionary process. Firstly, it sequesters IGF-I and induces cell death and secondly it activates tPA and thus induces the proteolytic cascades, including activation of MMPs, involved in the second stage of tissue remodelling in the mammary gland. In summary, we were unable to show any direct effect of IGFBP-5 on PAI-1 activity in IGF-independent way. Rather, the ability of IGFBP-5 to counteract the effects of PAI-1 appeared to be indirect, due to activation of tPA but not uPA. This does not rule out the possibility that IGFBP-5 can influence other actions of PAI-1 such as its ability to modulate cell migration involving a plasmin-independent process (Andreasen et al., 2000; Imai et al., 2000).

Chapter VI. General discussion

The primary aim of this study was to establish an *in vitro* cellular model that was relevant to the adult mammary gland and which could be used to test the regulation of IGFBP-5 expression and the function of the binding protein on cell death/survival and tissue remodelling.

We selected the mouse mammary epithelial cell line, HC11, which has been used extensively as a model of mammary differentiation. We have already noted that, although these cells can be induced to differentiate sufficiently to express β-casein, they express this at extremely low levels and do not express other caseins, and are, therefore, probably representative of the earliest stages of differentiation. Nevertheless, we considered HC11 cells to provide a useful comparison for the changes occurring between day 18 of pregnancy and day 1 of lactation, which DIP treatment attempts to mimic. Initially, it was very important to characterise the expression of the IGFBP profiles in these cells as this had not been done previously. Our main findings were that differentiating HC11 cells using DIP treatment up-regulated their secretion of IGFBP-5 protein by up to 10-fold and that, during this process, IGFBP-2 secretion was down-regulated. Although we were not able to identify a proteolytic activity towards IGFBP-5 or -2 in medium conditioned by either undifferentiated or differentiated HC11 cells, we did observe IGFBP-5 fragments on Western blots of HC11 conditioned medium, possibly suggesting that any proteolytic activity was ECM or cell surface associated.

However, despite considerable evidence from the literature that changes in the expression of both IGFBP-5 and -2 are associated with differentiation in a wide variety of cell types, with the former being up-regulated and the latter down-regulated, our more detailed analysis of the individual or pair-wise effects of the hormones D, I or P on the expression of these two genes clearly indicates that both IGFBP-5 and -2 expression can be dissociated from HC11 cell differentiation. For example, pair-wise combinations of D, I or P could induce IGFBP-5 expression without having any effect on β-casein levels, whereas dexamethasone alone was inhibitory for IGFBP-2 secretion, while prolactin actually stimulated the secretion of this protein. Furthermore,

the fact that substantial quantities of IGFBP-5 were secreted on day 6 of culture in undifferentiated HC11 cells further indicates that differentiation and IGFBP-5 secretion profiles can be disconnected. Although we have clearly demonstrated that the IGFBP-5 expression profile in HC11 cells is independent of the state of cellular differentiation, we are still very interested to know whether these cells up-regulate IGFBP-5 secretion under conditions of apoptosis and this is the subject of further investigation within our group.

We have also addressed the important question as to whether the epithelial HC11 cells provide an accurate model for the different developmental stages in the adult mammary gland with respect to the mRNA expression pattern of other members of the IGF axis. Our group has carried out extensive quantitative RT-PCR analyses to examine this and our findings are presented earlier in this thesis and in (Boutinaud et al., 2004). In general, we found parallels and differences between our *in vitro* studies and those occurring *in vivo*. In agreement were the low levels of expression of IGF-1, IGF-II and IGFBP-1 in both systems, which is to be expected, as the IGFs are derived from the mammary stroma and IGFBP-1 has not been shown to be expressed in either the stroma or epithelium of the gland. Also in agreement was the moderate level of expression of IGFBP-2 in both HC11 cells and mammary tissue, whereas IGFBP-3 and -5 were expressed at orders of magnitude higher in HC11 cells than that in the differentiating mammary gland. However, the most intriguing differences were that the expression profile of IGFBP-2 went in opposite directions during DIP treatment of HC11 cells and differentiation of the mammary gland and that although mRNA for IGFBP-4 and -6 was undetectable in HC11 cells, there were significant levels of both during involution of the gland and during late pregnancy for IGFBP-6. As a consequence, we believe that the differential regulation of IGFBP-2 between HC11 cells and the mammary gland and the site of expression of IGFBP-4 and -6 in the gland are important areas for future investigation. Based on the above we considered HC11 cells to be a useful *in vitro* model for some, but not all, of the components of the IGF axis.

An example where HC11 cells could prove to be an extremely good *in vitro* model for the induction of IGFBP-5 expression observed in the involuting mammary gland, is the significant up-

regulation of IGFBP-5 expression that occurs upon DIP treatment of these cells. We hoped that this would allow us to potentially identify the important transcriptional regulators responsible for an induction of IGFBP-5 gene expression in mammary epithelial cells. We employed transient transfection of HC11 cells with a series of IGFBP-5 promoter deletion constructs spanning +120 bp of exon 1 to −3000 bp from the 5' flanking region of the mouse IGFBP-5 gene upstream of the luciferase reporter gene. Our initial experiments agree very well with those of others in Hep G2 cells, where the construct with the largest stretch of promoter sequence had the lowest luciferase activity, while the region spanning positions −1004 to −156 resulted in the greatest activity. This suggests that the same transcriptional regulatory elements could be active in both HC11 and Hep G2 cells, and may even be used to control IGFBP-5 expression in a wide variety of cell and tissue types.

The studies presented in my thesis have shown that there is an enhancer element(s) between positions −1004 to −156 in the IGFBP-5 promoter that results in significant induction of gene expression in HC11 cells. In an attempt to identify the region containing this novel enhancer(s), we used site-directed mutagenesis and restriction enzyme digestion to reduce the region under examination to positions −556 to −156. This produced a complex result, which can best be explained by multiple regulatory elements lying on either side of the −556 position. However, we were most surprised to discover that mutagenesis alone, without subsequent deletion, also resulted in a lower luciferase activity in our assays. The only explanation that we can offer for this result is that we may have inadvertently interrupted a regulatory transcriptional binding site when we made our base pair substitutions and a computer search of this site identified potential transcriptional sites for Hox-1.3, a GC box factor known as SP1F, the zinc finger transcriptional factor (ZBP-89) and the MYC-associated zinc finger protein related transcriptional factor. However, none of these transcriptional factors have previously been shown to regulate the expression of IGFBP-5.

Irrespective of whether the above four transcription factors are involved in IGFBP-5 regulation or not, these findings are very significant, because, to date, all of the active regulatory

sites in the IGFBP-5 promoter have fallen within the first 71 bp downstream of the transcriptional start site (described in detail in Chapter I). This means that we have shown that there is a region much further downstream in the IGFBP-5 promoter, which contains a completely novel regulatory element(s) for directing IGFBP-5 expression in HC11 cells, and which may, therefore, by inference, may be active in the mammary gland *in vivo*. We believe that this finding strongly merits further investigation.

Finally, with respect to *in vitro* functional studies of IGFBP-5, we were able to examine the role of IGFBP-5 in modulation of plasmin generation. Our interest in a possible role for IGFBP-5 in plasmin generation came from publications arising several laboratories undertaking research in this area. Firstly, our own group had demonstrated that plasmin generation was increased in the mammary glands of transgenic mice over-expressing IGFBP-5. Coupled with the fact that we had shown that IGFBP-5 binds to αs2-casein whilst others had shown that this milk protein also binds tPA and plasminogen, the evidence became even more compelling. Finally, the observations of Clemmons group in the US, which identified a physical interaction between IGFBP-5 and PAI-1, provided us with the stimulus to pursue the biological relevance of this interaction. Given that IGFBP-5 increased plasmin generation, we began with the hypothesis that, if IGFBP-5 binds to PAI-1 then it serves to inhibit PAI-1 activity.

We took advantage of our *in vitro* culture of HC11 cells because they made sufficient concentrations of endogenous plasminogen activators to induce major morphological changes in overnight cultures and they were able to generate significant amounts of plasmin, capable of being used in a quantitative, biological assay for PAI-1. These studies quickly established that IGFBP-5 could indeed prevent the effects of PAI-1 and thereby induce increased plasmin generation *in vitro*. However, our simple hypothesis appeared to be not so simple when we demonstrated that a mutant form of IGFBP-5, which did not bind to heparin, was still biologically active in preventing the effects of PAI-1. This was unexpected since PAI-1 has been described as interacting with IGFBP-5 through its heparin-binding domain. Indeed, using Biosensor analysis we were able to show that whilst *wt* IGFBP-5 bound to PAI-1 the mutant which did not bind to heparin also did not bind to PAI-1. We therefore had to identify an alternative explanation for our results and were able to

conclude, and demonstrate, that IGFBP-5 was actually enhancing the effect of tPA but not uPA. tPA is classically associated with generation of plasmin in solution whereas uPA, bound to the uPA receptor, is involved with generation of plasmin at the surface of cells. tPA is thus considered an important protease activator in biological fluids such as blood and is indeed used as a "clot-buster". Our data therefore suggests that IGFBP-5 may play a role in modulating the important process of blood coagulation and this effect is thus worthy of future studies in systems of relevance to cardiovascular biology.

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