A MUTAGENIC AND BIOLOGICAL STUDY OF RAT INSULIN-LIKE GROWTH FACTOR-BINDING PROTEIN-2

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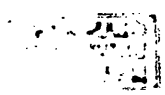
DECLARATION

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

Silvia Bramani



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ALS	acid labile subunit
BSA	bovine serum albumin
CM	conditioned medium
Cpm	counts per minute
DSS	disuccinimidyl suberate
ECL	enhanced chemiluminescence
ECM	Extra cellular matrix
FBS	foetus bovine serum
GAGs	Glycosaminoglycans
GH	growth hormone
HOX	homeobox gene
HRP	horse radish peroxidase
IGFBP	Insulin like binding protein
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor –I
IGF-IIR/Man-6-P	IGF-II/ Mannose 6 phosphate receptor
IGF-IR	IGF-I receptor
IP	immune precipitate
IR	insulin receptor
IRS-I	Insulin-like growth factor receptor substrate-I
kDa	kilo Dalton
Mr	Molecular weight
MTT	3-[4,5-Dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue
NBS	new-born calf serum
SDS-PAGE	sodium dodecyl sulphate polycrylamide gel electrophoresis
SFM	serum free medium
RT	room temperature
TBS-T	Tris buffer saline-Tween
wt	Wild type
P20A, P22A	IGFBP-2 mutants whose proline residues at positon 20 or 22 where substituted with an alanine
PGD-IGFBP-2	IGFBP-2 mutant in which RGD consensus sequence for integrin recognition was sustitued with PGD

Summary

We have undertaken a mutagenic and cell biological study of the properties of the rat insulin like growth factor binding protein-2 (IGFBP-2). This 32kDa protein belongs to a family of six high affinity IGFBPs (IGFBP 1-6) which are well characterised in both rodents and humans.

In initial studies we mutated two highly conserved proline residues in the N-terminal region of IGFBP-2 to create the mutant proteins P20A-IGFBP-2 and P22A-IGFBP-2 and expressed these proteins along with wild type rat IGFBP-2 in the baculovirus/insect cell system. We found that this expression system was able to produce correctly folded, functional rat IGFBP-2 and using solution phase equilibrium binding techniques we demonstrated that the affinity of these mutated proteins for both IGF-I and IGF-II was similar to that of the wild type protein. We conclude that, although highly conserved in all IGFBP species, these proline residues are not involved in the IGF ligand binding site of rat IGFBP-2.

In order to investigate the biological functions of IGFBP-2 in the regulation of IGF action we undertook experiments to establish a cell culture model in which the appropriate components of the IGF axis were present to allow such an investigation to take place. In initial experiments we screened several cell lines for the presence of cell or extra-cellular matrix associated IGFBP-2 (a phenomenon which limited reports in the literature had previously described). For one of these cell lines Clone 9 - a cell line derived from rat liver- we demonstrated clearly by Western blotting, ligand blotting and immunoprecipitation the association of IGFBP-2 with the surface of the cell line although experiments to determine the chemical nature of this interaction proved inconclusive. In addition to the demonstration of cell membrane associated IGFBP-2 we also identified the presence of IGF-I receptor on these cells and we were also able to show that these cells secrete IGF-I (but not IGF-II) into the surrounding medium.

The identification of these components of the IGF axis in Clone 9 cells encouraged us in biological experiments to examine the possible modulatory role of cell-membrane associated and secreted IGFBP-2 on the modulation of IGF-I activity at the IGF-I receptor. We initially established that Clone 9 cells do indeed respond to exogenous IGF-I both in short term signalling events (acute tyrosine

phosphorylation of a 180kDa protein) and in longer term metabolic/mitogenic assays as determined by MTT-formazan dye conversion.

Further, we demonstrate, by the use of the IGF-I analogue des(1-3) IGF-I (which does not interact with IGFBPs but retains wild type potency at the IGF-I receptor) that IGF-I activity is inhibited both acutely and in longer term assays by the presence of IGFBP-2. We postulate that the former inhibition may be due to the association of IGFBP-2 with the plasma membrane of Clone 9 cells while the latter may be more related to the accumulation of IGFBP-2 in the conditioned medium.

In order to further test and clarify this hypothesis we finally report on some preliminary experiments aimed at stably transfecting Clone 9 cells with sense or antisense IGFBP-2 constructs. Initial characterisation of mixed populations of such cells show no difference in basal growth rates between control, antisense and sense transfected cells although we observed reduced secretion of IGFBP-2 into conditioned medium by antisense transfected cells. We believe that the use of these cells will aid in the clarification of the role of IGFBP-2 in the regulation of the autocrine and perhaps paracrine role of IGF-I.

In the final chapter of this thesis we expand on those areas of the current work which we feel would benefit from further research.

OVERVIEW

Insulin–like growth factors (IGFs) were first discovered while investigating the mode of action of pituitary growth hormone (GH) (Salmon and Daughaday, 1957). Many of the *in vivo* growth effects attributed to GH were demonstrated to be not due to the direct interaction of GH with its own receptor, but the result of the GH-stimulated increase of a circulating plasma factor, IGF-I, which subsequently exerted potent metabolic and mitogenic effects at the tissue level. For this reason IGF-I is often referred to as somatomedin (i.e. mediator of somatotropin, which was the name traditionally attributed to GH) (Van Wyk *et al.*, 1974). Since they were discovered, many studies have been undertaken to characterize IGF molecular features and to highlight the complexity of their regulation and the multiplicity of their functions.

There are two IGF peptides, IGF-I and -II, which share similar structure and which are related to insulin. IGF-I and II are both widely expressed in the tissues but they show a distinct hormonal regulation, tissue specificity and dependence on physiological status. Both IGF-I and -II seem to exert their physiological action through the cell membrane IGF-I receptor, but they can also bind IGF-II and insulin receptors. The local concentration of IGFs and the abundance of the receptors on the cell membrane are not the only factors that influence IGF biological action as the actual bioavailability of the peptides also plays a major role. In fact in the plasma and tissues only a small percentage of IGFs are present in a free form with the bulk of hormone complexed with a family of IGF-binding proteins (IGFBPs).

The IGFBP family comprises 6 well characterised members each of which binds IGF-I and -II with a different affinity. IGFBPs can be found ubiquitously in the organism, although they have a different tissue specificity and regulation of gene expression.

When IGFBPs were first identified in the plasma they were believed to function mainly as carrier proteins as it was shown they significantly increase IGF half-life in blood and regulate IGF distribution to the tissues. Lately when IGFBPs were found to be expressed in virtually all body tissues and *in vitro* cell cultures it became the general opinion that IGFBPs exerted an inhibitory effect on IGF action by sequestering the peptides from interaction with IGF-I receptor.

However as research continued, an increasing number of studies reported that in some cell systems IGFBPs displayed an IGF enhancing effect. This observation implies that, although IGFBPs generally reduce IGF bioavailability, in some cases IGFBPs may somehow augment IGF's interaction with its receptor. It became clear that the *in vitro* determined affinity constants of IGF for IGFBPs or for the receptor might not give a real picture of what happens in a biological context. In fact the association of IGFBPs with the cell surface or the extracellular matrix (ECM), the presence in biological fluids and cell conditioned media of specific IGFBP proteases and the occurrence of postranslational modifications of IGFBP molecules can significantly affect the IGF-binding affinity and therefore have a considerable impact on IGF action. Many more questions still need to be answered in order to highlight the precise mechanism by which IGFBPs dynamically modulate IGF bioavailability. In addition, the well reported IGF-independent effects that IGFBPs seem to exert by directly interacting with cell membrane proteins is also worth further investigations.

The apparent redundancy of six IGFBPs for one common function (i.e. binding IGFs) is unusual and alternative functions have been postulated. One hypothesis is that each IGFBP member, at the molecular level, plays a specific biological role. Alternatively the final IGFBP biological effect could result from a reciprocal modulation between IGF, IGFBP and the environment and therefore the same IGFBP may exert different actions in different biological contexts. Additional information on the molecular features of IGFBPs and on the IGF-IGFBP-environmental axis will be important for understanding the mechanism involved in the widely described but never elucidated IGF enhancing effect of IGFBPs. Again, the existence of two IGFs and two IGF receptors might look unnecessary duplication as both growth factors seem to signal only through the IGF-I receptor.

These are just some aspects of the complexity of the IGF-IGFBP axis which stimulated our interest and led to our work into this research area.

1. INTRODUCTION

1.1 Insulin-like growth factors (IGFs)

Insulin-like Growth Factors, IGF-I and IGF-II, are small polypeptide hormones structurally and functionally related to insulin. Despite many similarities with insulin, IGFs show a distinct biological action and play specific roles in the organism.

1.1.1 IGF STRUCTURE

IGFs are single chain, basic polypeptide hormones of about 7.5 kDa (Daughaday and Rotwein, 1989; Rotwein 1991). The mature protein, based on the similarities with insulin, can be divided into 4 distinct domains:

- A and B domains correspond to the a and b chains of the mature insulin molecule The B domain contains the N-terminus of the IGF molecule. The A and B domains of IGF-I and II share 70% identity, while the overall amino acid sequence homology between IGF A-B domains and insulin a-b chains is as high as 50% (Phillips *et al.*, 1998).
- a 12 residue C domain (8 residues in IGF-II) which links the A and B domain and corresponds to the peptide that connects the a and b chain in pro-insulin. (De Meyts *et al.*, 1994)
- and a 8 amino acid D domain (6 residues in IGF-II) which represents the C-terminus of the IGF molecule (De Meyts *et al.*, 1994).

In addition some incompletely processed high Mr IGF-I and II have been described. A 12-18 kDa pro-IGF-I retains part of the signal peptide (Goldstein and Phillips, 1989; Acquaviva *et al.*, 1982; Goldstein and Phillips, 1991). Instead the so-called "big IGF-II" is an immature IGF-II molecule with a carboxyl terminal extension due to the incomplete cleavage of the E domain that is present in pro-IGF-II. A big IGF-II form referred to as (E1-21) is one of the factors which is associated with the appearance of hypoglycemia in non islet cell tumors (Hizuka *et al.*, 1998).

IGF and insulin are also very similar in 3-D structure and they have 3 similar disulfide bridges (De Meyts *et al.*, 1994).

In Fig *1.1.1* the tertiary structure of IGF-I, proinsulin, insulin and relaxin, all members of insulin/IGF peptide hormone family is compared (Fig from De Meyts *et al.*, 1994, with permission).

1.1.2 IGF RECEPTORS

Two IGF receptors, IGF-IR and Man6P/IGF-IIR are present at the cell surface, but most of the biological actions attributed to IGF-I and -II appear to be mediated by the IGF-IR

1.1.2a Type 1 IGF-I receptor or IGF-IR

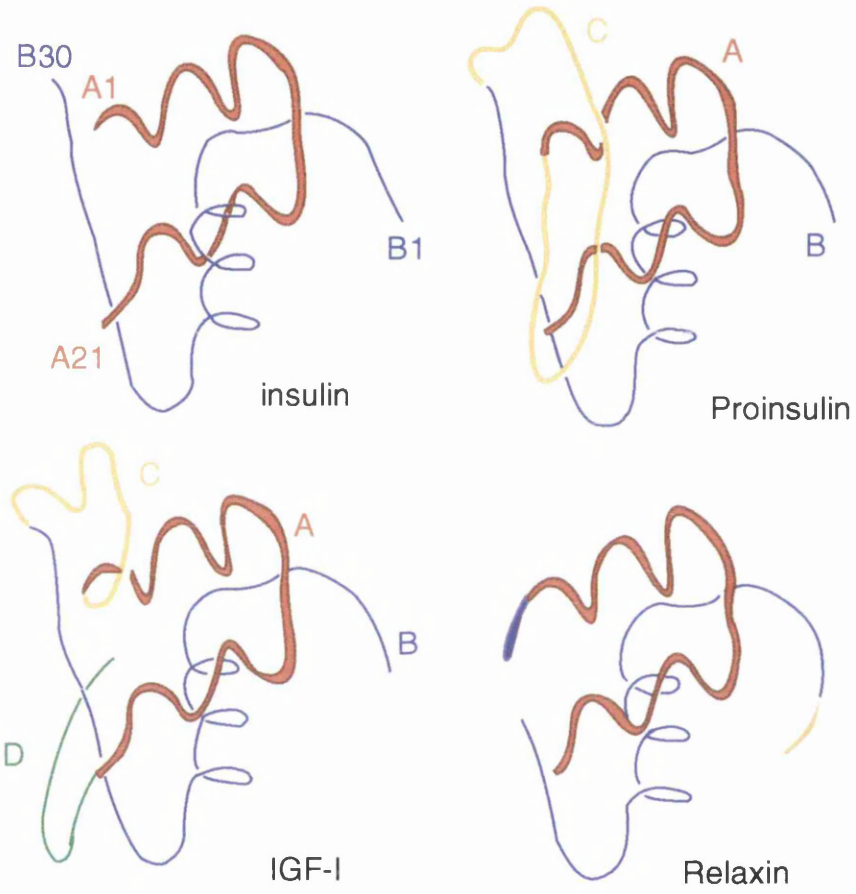
IGF-I receptor (IGF-IR) has a wide distribution in tissues and cell cultures (as has the insulin receptor, IR). It is believed that IR has a predominant metabolic effect, while IGF-IR is mainly mitogenic/transforming/antiapoptotic (Sepp-Lorenzino, 1998). i) The structural similarity between the two receptors; ii) the fact that they coexist in many cell lines; iii) the possibility that insulin or IGF-I cross-reacts with IGF-IR or IR respectively; iv) the naturally occurring formation of IR-IGF-IR hybrids and v), the overlapping pathways activated by the two receptors are some of the difficulties involved in trying to identify where insulin and IGF-I receptor signals diverge.

IGF-IR binds IGF-I with high affinity (k_d 10^{-9}M), IGF-II with 10 fold lower affinity and it can also bind insulin weakly (with 500-1000 fold lower affinity than for IGF-I) (Nissley *et al.*, 1991). IGF-I receptor is an $\alpha_2\beta_2$ heterotetrameric glycoprotein, very similar to the insulin receptor (IR) (De Meyts *et al.*, 1994). The extracytoplasmic α subunit (Mr 130kDa) represents the ligand binding domain and it is disulphide bonded to the β subunit (Mr 95kDa) which is responsible for signal transduction (Moss and Livingston, 1993; Alexandrides *et al.*, 1993). The β subunit is formed by a short extracellular domain, a single membrane-spanning domain, a juxta-membrane domain, an ATP binding site and a tyrosine kinase domain. Extensive mutagenic studies of the β subunit led to the identification of sequences and domains which are specifically involved in either the mitogenic, transforming or antiapoptotic function of the receptor or identified regions required for IGF-IR internalization after ligand binding (Sepp-Lorenzino, 1998).

Unlike other growth factor receptors, IGF-IR is a constitutive dimer (De Meyts *et al.*, 1994), being formed by two functional α-β halves linked to each other by disulphide bridges. The receptor activation only occurs when the 2α subunits are bridged through the ligand molecule. There are 2 binding

Fig *1.1.1*

Comparison of tertiary structure of members of insulin/IGF peptide hormone family



Computer modeling of tertiary structure of insulin, proinsulin, IGF and relaxin. These peptides together with IGF-II, invertebrate bombexin and molluscan insulin-like peptide form a family of peptide hormones.
(From De Meyts *et al.*, 1994, with permission)

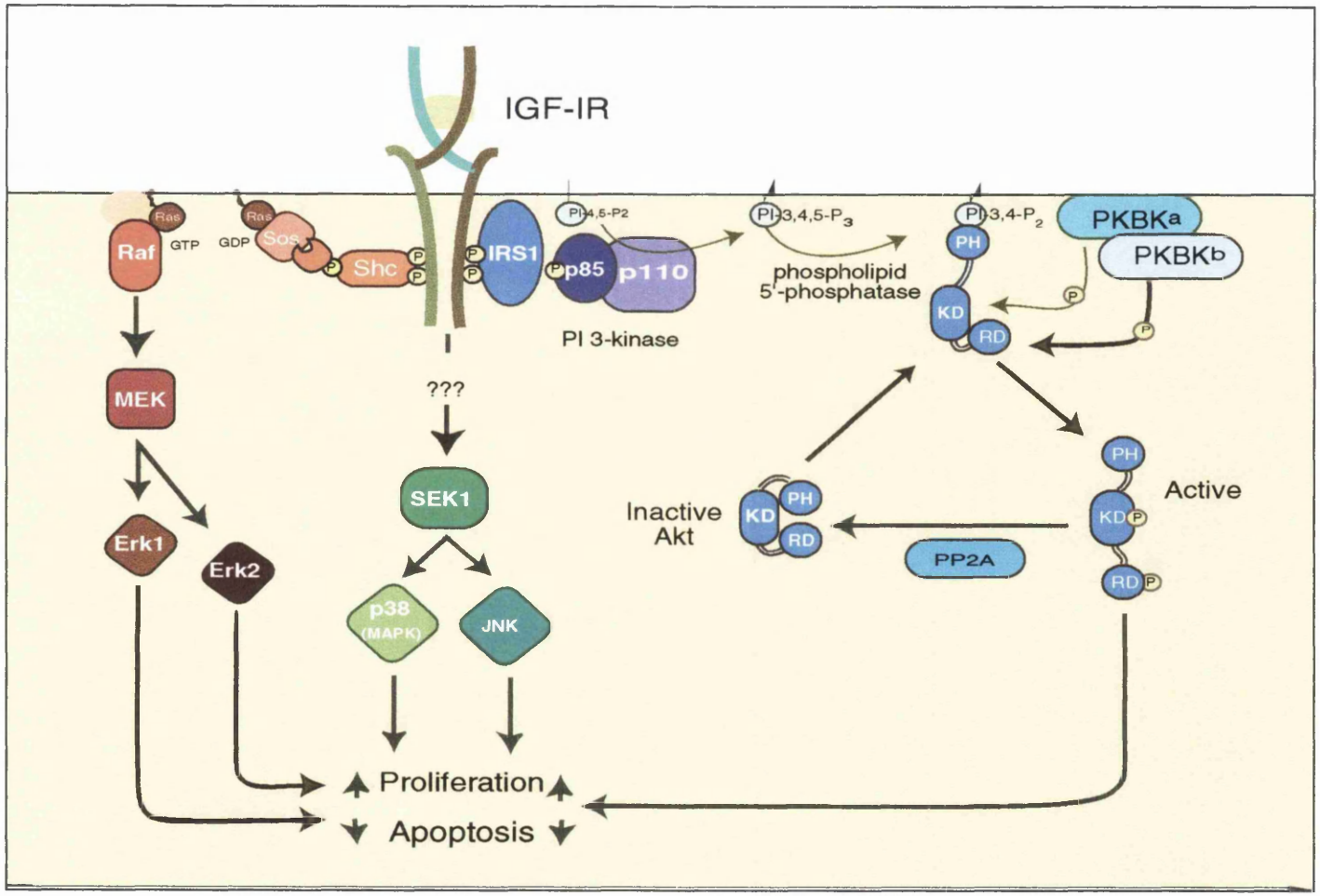
domains in each α subunit and each IGF-IR binds one IGF molecule with high affinity and a second one with 10 times lower affinity causing curvilinear Scatchard plots. The concentration of the ligand might modulate effectiveness of signalling as the binding of a second molecule to the receptor causes the release of the first ligand molecule in a negative cooperativity manner (De Meyts *et al.,* 1994). Ligand binding at the extracellular α subunit results in the activation of the tyrosine kinase domain of the β subunit which gives rise to a reciprocal β subunit transphosphorylation and to tyrosine phosphorylation of signal mediator proteins, such as IRS-I and 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. Probably the most important pathway through which IGF-I exert its mitogenic effects is the activation of the *ras-raf*-MAP kinase pathway. IGF-IR does not activate *ras* directly but the activated IRS-I, Shc and Crk bind Grb-2 protein which finally binds mSOS and the signal continues through *ras* and the MAP kinase pathway. Activated IRS-I also binds p85 subunit of the phosphatidyl-3 kinase (PI 3K) and stimulates the activity of p110 catalytic subunit of PI 3K. The PI 3 kinase pathways might be involved in the survival-promoting action of IGF-I activating Akt pathways (Dudek *et al.*, 1997). A diagram of IGF-I signal transduction pathways is shown in Fig. *1.1.2.a.*

As indicated above, through the activation of cytoplasmic protein mediators, the signal can spread horizontally leading to the simultaneous activation of multiple signal pathways which share common mediators. It is note-worthy that not only do many pathways share the same mediators, but that signal initiated from different receptors might be transduced through the same pathway and still produce different specific effects. Therefore in cells which express a variety of receptors there must be a way for each receptor to exert specific effects. It has been suggested that each receptor might activate a still unidentified characteristic mediator or perhaps may activate common mediators in a unique manner (Rubin and Baserga, 1995). Moreover De Meyts *et al.*(1994) suggest that the consideration of quantitative and time related aspects of signalling events might help to answer these questions.

1.1.2b Type 2 IGF receptor or IGF-IIR

The IGF-IIR is a monomeric, 260kDa glycoprotein homologous to the cation-independent mannose 6-phosphate receptor which is involved in trafficking of the lysosomal enzymes (Jones and Clemmons, 1995a; Sepp-Lorenzino, 1998). The IGF-II/Man-6-P receptor consists of 15 extracellular

Fig *1.1.2.a*
IGF-IR signal pathways (from Werner &LeRoith 1997, with permission)



contiguous repetitive domains (each of 150aa in length) (Marron-Terada *et al.*, 1998) and a short cytoplasmic domain which does not have tyrosine kinase activity. IGF-II/Man-6-P receptor is also present on other organelle membranes and its function as a carrier in the delivery of newly synthesized lysosomal enzymes is well known. The receptor situated on the membrane of Golgi vesicles binds the mannose residues of the newly synthesized lysosomal enzymes and delivers them to the endosomes. From there the receptor-enzyme complex reaches the lysosomes where the acidic pH allows the enzymes to dissociate from the IGF-II/ Mannose 6 phosphate receptor and the unoccupied membrane-bound receptor can then be recycled through the Golgi network (Brown *et al.*, 1986).

Two distinct forms of Man-6-P receptors (215kDa and 46kDa as described in Clone 9 rat liver cells) are present on the cell surface and become internalized after binding Man-6-P containing proteins, but only the 215kDa form is able to bind IGF-II (Matovcik *et al.*, 1990).

The binding, to IGF-IIR/Man-6-P, of some ECM glycoproteins, or renin (Faust *et al.*, 1987) proliferin (Lee and Nathans, 1988), thyroglobulin (Herzog *et al.*,1987) and the latent form of transforming growth factor-β (TGF-β) (Purchio *et al.*, 1988) has been reported. In this last case the latent form of the TGF-β after binding to IGF-IIR/Man-6-P receptor is cleaved and becomes active (Dennis and Rifkin, 1991).

Cell membrane IGF-II/Man-6-P receptor binds IGF-II peptide with high affinity (0.017-0.7nM) (Nissley *et al.*, 1991), has 500-1000 fold lower affinity for IGF-I and it does not bind insulin (Jones and Clemmons, 1995a). Man-6-P and IGF-II are competitors for binding to the receptor. After ligand binding has occurred the membrane bound receptor is internalized and IGF-II degraded at a lysosomal level (Oka *et al.*, 1985). The extracellular portion of the receptor can be proteolytically cleaved and reaches the circulation. Whether or not this circulating molecule has a role in the regulation of IGF-II is not clear (LeRoith, 1996).

The biological function of IGF-II/Man-6-P receptor also remains unclear. There is a general agreement on its function as a scavenger receptor. In IGF-IIR knock out mice 100% of foetuses died at 15 days of gestation (Lau *et al.*, 1994; Wang *et al.*, 1994). When IGF-IIR and IGF-II genes are both deleted some of the foetuses carried on gestation till birth and one pup reached adulthood. These data seem to indicate that at least in the foetus IGF-II clearance through IGF-II receptor is essential (Filson *et al.*, 1993).

It is also believed that most of the biological functions attributed to IGF-II are mediated through the IGF-IR. Nevertheless, there are some reports on IGF-II actions mediated through the IGF-IIR. In a rat

neuroblastoma cell line which expresses both IGF receptors and responds to IGF-I or II with an increased cell proliferation, IGF-I treatment also increased the secretion of IGFBP-4 into the conditioned medium. In contrast IGF-II decreased IGFBP-4 secretion by 50%. Treatment of the cells with anti IGF-IIR antibody alone caused a 70% inhibition of IGFBP-4 secretion and the effect was greater if IGF-II was present at the same time. These authors conclude that in this cell line IGF-II acts in part through IGF-I R and in part through IGF-II R (Ceda *et al,* 1991). A signal pathway has never been identified for IGF-II/Man-6-P receptor, but the receptor is reported to be coupled with GTP binding proteins. When IGF-II binds to the receptor it stimulates the binding of GTPγS to G_{i2} and inhibits the GTPase activity. The observed Ca^{++} influx stimulated by IGF-II in 3T3 fibroblasts could be mediated through IGF-II/Man-6-P receptor and the coupled GTP-binding proteins (Nishimoto *et al.*, 1987 and 1989; Murayama *et al.*, 1990; Kojima *et al.*, 1988). Recently it has been reported that IGF-II promotes exocytosis of insulin in pancreatic cells through Man-6-P/IGFIIR. This effect is independent from Ca^{++} influx and is mediated by the phosphorylation of protein kinase C (Zhang *et al.*, 1997).

1.1.3 IGF FUNCTIONS

Many similarities between IGF and insulin functions can be reasonably expected considering the already mentioned molecular homology between the peptides and their receptors. In addition it has been shown that the two receptors can form IR-IGF-IR hybrids *in vivo* (De Meyts *et al.*, 1994) and that a low affinity cross-binding between IGF-I and IR or insulin and IGF-IR occurs when the ligands are present at high concentration. Furthermore once activated the two receptors seem to share common mediators of signal transduction. Despite these considerations it must be pointed out that IGF and insulin have developed specific biological roles.

Insulin is a typical endocrine factor. It is produced by β cells in the pancreas then enters the circulation and reaches the target organs -mainly liver, muscle and adipose tissue- where it stimulates anabolism via glucose and amino acid uptake.

IGF-I has an endocrine mode of secretion in the liver where it is primarily under GH control and this provides the main source of circulating IGF-I. Typical of an endocrine hormone is also the negative

regulation of pituitary GH secretion that is triggered by increased IGF-I plasma concentration (Phillips *et al.*, 1998). Conversely, unlike typical endocrine hormones, IGF-I is also expressed in many organs and tissues in an autocrine/paracrine fashion. In some cases as in the epiphyseal growth plate (Jennische *et al.*, 1992), in the ovary (Giudice, 1992) and kidney (Chin *et al.*, 1992) this local production is under GH regulation, while in other cases, such as in the uterus (Murphy *et al.*, 1987), IGF-I expression is GH-independent. In some pathological states GH regulation of hepatic IGF-I production can be overcome by inhibitory factors. An abnormally low plasma insulin concentration or a restricted availability of energy and protein (in particular essential amino acids) negatively regulate IGF-I despite normal levels of GH (Phillips *et al.*, 1998). The design of the IGF-I gene allows the alternative usage of promoters and initiation transcription sites, includes multiple sites of polyadenylation together with alternative splicing and variations in mRNA stability offering many opportunities for the regulation of IGF-I production (Phillips *et al.*, 1998).

Unlike IGF-I, IGF-II secretion occurs in an autocrine/paracrine GH-independent mode (Jones and Clemmons, 1995a). IGF-II is the predominant IGF in fetal life, after birth its concentration decreases while GH concentration rises and stimulates a concomitant increase in IGF-I. In rat, IGF-I becomes the major species in adult plasma (Phillips *et al.*, 1998) while in humans IGF-II is the predominant hormone (Gargosky *et al.*, 1990; Suikkari and Baxter, 1992). In the fetus IGF-I is GH independent and its responsiveness to GH becomes gradually effective in postnatal life (Jones and Clemmons, 1995a).

IGFs are involved in fetal growth and development. IGF-I, -II and IGF-IR are expressed in rat embryos as early as the 8-cell stage (Heyner *et al.*, 1989; Heyner and Garside, 1994). Targetted disruption of the IGF-I gene in mice produces a phenotype of 60% weight at birth, variable postnatal lethality and delays in ossification (Baker *et al.*, 1993), whereas IGF-II knockout mice are 60% smaller than normal pups, but are otherwise proportionate and vital (DeChiara *et al.*, 1990; DeChiara *et al.*, 1991). Homozygous IGF-IR deficient mice only weigh 45% of normal mice. They present abnormalities in various organs and die at birth (Liu *et al.*, 1993). The disruption of IGF-I and IGF-IR genes did not further reduce the weight of the embryos. In contrast when the IGF-II gene was disrupted in combination with either IGF-I or IGF-IR genes the weight of the embryos dropped to 30% indicating that some of the growth promoting effect of IGF-II is not mediated through IGF-IR (Liu *et al.*, 1993). IGF-IIR knock out mice die in utero, but the

double suppression of IGF-IIR gene and IGF-II gene rescues the mice which show 60% of normal weight at birth and are mostly viable (Filson *et al.*, 1993).

1.1.3a The in vivo biological effects of IGFs

are mainly:

- an anabolic insulin-like effect (stimulation of glucose uptake and glycogen synthesis) on fat and muscle cells (Froesch *et al.*, 1985)
- a growth promoting effect consisting essentially in mediating the GH-stimulated body weight gain and skeletal elongation (Salmon and DuVall, 1970)
- stimulation of cell proliferation in a variety of organs and tissues (Davoren and Hsueh, 1986; Davoren *et al.*, 1985; Kasson and Hsueh, 1987; Rechler *et al.*, 1976; Conover *et al.*, 1985).

1.1.3b In vitro IGF action

can be summarized as:

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

- Stimulation of cell differentiation in osteoblasts (Sara and Hall, 1990), chondrocytes (Geduspan and Solursh, 1993), adipocytes and neural cells (Pahlman *et al.*, 1991)
- Induction of an acute insulin-like effect on protein and carbohydrate metabolism, especially in myoblasts (Dimitriadis *et al.*, 1992)
- Regulation of hormone secretion or induction of chemiotactic migration in specific cell types.(Stracke *et al.* 1989; El-Badry *et al.*, 1990).

The wide range of IGFs actions is controlled by the availability of the hormones and their receptors. Many factors can affect this equilibrium but it is believed that a major role is played by a family of IGF binding proteins.

1.2 Insulin-like growth factor-binding proteins (IGFBPs)

The IGF binding proteins (IGFBPs) constitute a family of 6 well characterized proteins that bind IGFs with high affinity, but do not bind insulin. IGFBPs are identified with a progressive number from 1 to 6 which represents the chronological order of their discovery. IGFBP Mr ranges between 24 and 40 kDa and all members share many similarities in terms of molecular structure, IGF-binding, biological function, gene organization and evolution.

The structure and the functions of insulin-like growth factor binding proteins have been reviewed by Jones and Clemmons (1995a) in a comprehensive and detailed manuscript. It is the intention of the present introduction to give special emphasis to the literature published in the last four years.

Recently a new group of low affinity IGF binding proteins has been described which comprises mac25, CTGF, *nov,* and *cyr*61. As these proteins share some structural similarities with IGFBPs and can bind weakly to IGFs, they were initially named IGFBP-7, 8, 9 and 10 respectively. However at the fourth IGF Symposium (Tokyo) it has been recommended to group these proteins in the IGFBP superfamily under the heading of *IGFBP-related proteins*. Further elucidation of the molecular and biological features of these proteins is necessary for their definitive classification (Baxter *et al.*, 1998).

For simplicity, only the 6 high-affinity IGFBP members will be considered in this introduction while characteristics of the IGFBP-related proteins compared to IGFBP (1-6) will be described separately.

1.2.1 IGFBP STRUCTURE

Analyzing the aligned amino acid sequences of the 6 IGFBPs it appears that they all have 16-18 cysteine residues in conserved positions. Based on cysteine distribution and on sequence homology, the IGFBP molecule can be divided in 3 distinct regions (Fig *1.2.1*):

- an ***N-terminal region*** which typically contains 12 cysteine residues (exceptions are human and rat IGFBP-6 that lack 2 and 4 Cys residues respectively) (Shimasaki *et al.*1991)
- a ***central region*** which is virtually cysteine-free (except in human, bovine and rat IGFBP-4 which contain 2 Cys (Landale *et al.*, 1995).and
- a ***C-terminal region*** which contains the remaining 6 cysteines.

The N terminal and C terminal regions are well conserved among different classes of IGFBPs and between different animal species (50% and 80% homology respectively). The conserved homology suggests that these two regions might be structurally/functionally essential for the biological activity of IGFBPs. The observation that both the N terminus and the C terminus of IGFBPs are involved in IGF-binding and that the C-terminus is necessary for the association of IGFBPs with the cell surface or the extracellular matrix, seems to support this hypothesis.

In contrast the central region is not conserved among different IGFBPs and it is considered as a *hinge domain* which connects the N- and C-terminal ends of the molecule. The central region of some IGFBPs is a target of specific IGFBP proteases which regulate IGFBP bioavailability (Lee *et al.*, 1997; Clemmons, 1997; Zheng *et al.*, 1998a). A schematic diagram of IGFBP general structure is shown in Fig. *1.2.1.*

Fig *1.2.1*

3 domain structure of IGFBPs

N- terminal region- 12 Cys
C- terminal region- 6 Cys
} high structural homology

Central region-Cys-free, no sequence homology

The high number of Cys residues indicates that IGFBPs are probably highly structured and that the 3-D structure might be important for the functionality of these proteins. It is believed that most, if not all, the cysteines are engaged in disulfide bridges. The demonstration that the 18 cysteines in hIGFBP-3 (Sommer *et al.*1991) and at least 16 cysteines in hIGFBP-1 (Brinkman *et al.*, 1991a) are disulphide bonded supports this assumption. Links between Cys^{56}-Cys^{69} and Cys^{63}-Cys^{89} were identified in rat IGFBP-3 with peptide mapping procedures (Hashimoto *et al.*, 1997). Recently Kalus *et al.*(1998) analyzed IGFBP-5 region between Ala^{40} and Ile^{92} and concluded that the formation of disulphide bridges between Cys ^{47}-Cys^{60} and Cys^{54}-Cys^{80} are the most likely to form and they correspond exactly to the cysteine pairs reported by Hashimoto in IGFBP-3.

However it was not clear whether the disulphide links were established intra domain or if they cross-linked the N terminus and C terminus domains. The observation that it was possible to isolate the N-terminal region of the IGFBP molecule without reduction of the disulfide bridges suggested that the N-terminal and the C-terminal regions were not linked by S-S bridges (Sommer *et al.*, 1991). Recently this result has been confirmed by Forbes *et al.* (1998) using CnBr digestion and SDS-electrophoresis under non reducing conditions. This indicated that none of the Cys residues of the N terminus of IGFBP-2 is engaged in an interdomain disulphide bridge. In addition the authors assigned the S-S bond pattern in the C-terminus of bIGFBP-2 as (Cys^{186} -Cys^{220} ; Cys^{231} -Cys^{242}; Cys^{244}-Cys^{265}) showing that all 6 C-terminal Cys residues are engaged in disulphide bonds within the same domain.

An almost contemporary paper showed that no disulfide bonds linked the N-terminal and C-terminal regions of hIGFBP-6 and provided also a partial identification of the disulfide bridge pattern (Neumann *et al.*, 1998). In this work hIGFBP-6 fragments resulting from a partial digestion with trypsin or chymotrypsin were purified by reverse-phase HPLC under denaturing conditions (6M urea and 10mM DTT), but unexpectedly these reducing and denaturing conditions did not break S-S bridges and allowed the purification of disulfide bonded peptides. The authors propose that the location of disulphide bonds redefines the 3-domain structure and rearranges the conformation of IGFBP-6 within 4 subdomains, 2 in the N-terminus and 2 in the C-terminus The first subdomain includes the first 6 cysteine residues (29, 32, 40, 44, 57, 63) of N-terminal IGFBP-6 which appear to be all engaged in 3 disulfide bridges. The second subdomain results from the connection of $Cys^{104}$with either $Cys^{71,}$ Cys^{78}or Cys84; the C-terminus third

subdomain is structured around a Cys^{163}-Cys^{190} bridge and the last subdomain is formed by the link between either Cys^{201} or Cys^{234} with Cys^{212} or Cys^{214}.

Before the publication of these data, the lack of information on IGFBP crystallographic structure made investigation of the molecular functional domains of the IGFBPs extremely difficult. In fact any cleavage or mutation of the IGFBP molecule could have interfered with the correct formation of disulphide bonds causing major changes in the tertiary structure and any loss of biological function of the modified IGFBP molecule could have made the results difficult to interpret. Instead Forbes and Neumann's work supports the idea that IGFBP tertiary structure folds separately within the N-terminus and the C-terminus forming two distinct domains whose structure and functions can be studied, at least in part, independently.

The high degree of sequence homology between IGFBPs of different animal species and among different IGFBP family members supports the hypothesis that all 6 IGFBP genes are derived from a common ancestral gene. IGFBP genes are closely linked to Homeobox (Hox) genes. HOX genes encode for transcription factors and are located on the same chromosome regions as the IGFBP genes. In humans IGFBP-1 and 3 and the HOX A cluster genes are located on chromosome 7; IGFBP2 and 5 and HOX D cluster on chromosome 2; IGFBP4 and HOX B cluster on chromosome 17; and finally IGFBP6 and HOX C cluster on chromosome 12 (Lee *et al.*1997). It is believed that originally a single copy of IGFBP and HOX ancestral genes were associated on the same chromosome before they underwent duplication and dispersion to the current chromosomal loci (Lee *et al.*1997). It has been suggested that after a first step of duplication and translocation the fate of the IGFBP-1-3 and 2-5 genes (all containing 18 Cys) diverged from IGFBP4 and 6 (which have 20 and 16 Cys, respectively). Subsequently other round of duplications and translocations gave rise to the current locations. It has been speculated that the duplication of a 2 gene-cluster gave rise to the current location of the IGFBP1-3 pair on chromosome 7 and the IGFBP 2-5 gene cluster on chromosome 2. The duplication of clustered genes might explain some structural and functional similarities between IGFBP1 and 2 and between IGFBP-3 and 5. The first two proteins share the presence in the molecule of an RGD sequence for integrin-binding and their concentration in serum is subjected to metabolic regulation, whereas IGFBP3 and 5 show high protein homology and are growth related (Collett-Solberg and Cohen, 1996). Despite the high degree of similarities each IGFBP developed specific features in relation to postranslational modification and functions.

IGF-RELATED PROTEINS OR LOW AFFINITY IGF-BINDING PROTEINS

Mac25 (product of mac25 cDNA), TAF (tumor-derived adhesion factor), PSF(prostacyclin-stimulating factor) are different names initially attributed to the same protein. Mac25 is structurally related to IGFBPs as it conserves 11 of the 12 cysteine residues present in the N-terminus of IGFBPs, and is capable of binding to IGF-I and -II, although with relatively low affinity. Due to these features mac25 was provisionally named IGFBP-7 (Oh *et al.*, 1996).

The connective tissue growth factor (CTGF), nov and cyr61 are instead three different proteins encoded by a family of immediate-early genes. As for mac25, the N-terminal region of these proteins are homologous to IGFBPs, while the C-terminus is not conserved. As CTGF expressed in baculovirus was demonstrated to bind IGF-I and –II with low affinity it was designated IGFBP-8 (Kim *et al.*, 1997). The remaining two proteins (nov and cyr 61) were only proposed as candidate members of the IGFBP family on the basis of their structural homologies (Baxter *et al.*, 1998).

However i) the lack of the cysteines in a conserved location in the C-terminal region of mac 25 (Hashimoto *et al.*, 1997); ii) the lower amino acid identity of mac25 with IGFBP (1-6) compared to the sequence homology among the 6 traditional IGFBP members; iii) the low binding affinity for IGF-I and -II displayed by mac25, iv) the ability of mac25 to bind insulin with high affinity, a property shared with the N-terminus of IGFBP-3,(Yamanaka *et al.*, 1997) and v) the location of mac25 gene on a chromosomal site which does not contain a HOX gene cluster suggests that further investigation is needed to enable a correct classification of the IGFBP-related proteins (Lee *et al.*, 1997).

Table 1.2.1 (based on Jones and Clemmons, 1995a; LeRoith, 1996; Hodgkinson *et al.*, 1994 and Swiss-Prot sequence data base)

Characteristics	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6
N residues						
human	234	289	264	237	252	249
rat	247	270	265	233	252	252
Mr theoretical						
human	27903	35137	31660	27934	28553	30570
rat	29628	32883	31680	27745	28428	30298
SDS-page	30	32	39-41	24-28	30	30
(non reducing) ≈						
phosphorylation	+	-	+	-	+	-
Glycosylation	-	-	N	N	O	O
RGD sequence	+	+	-	-	-	-
Heparin-bind	S	S	S + L	nd	S+S+L	Nd
(S= short; L=long)						
Other basic aa seq.			215-232		210-218	
Heparin binding	+/-	+	++	+	++	Nd
Cell surf. associat.	+	+	+	-	+	-
proteolysis	-	+	+	+	+	Nd
IGF-I-IGF-II						
preference	≈	≥IGF-II	≥IGF-II	≈	≥IGF-II	>>IGF-II
Chromosomal						
localiz. (human)	7p	2q	7p	17q	2q	12
HOX gene						
association	HOXA	HOXD	HOXA	HOXB	HOXD	HOXC

nd=not determined

1.2.1.1 PHOSPHORYLATION

As indicated in table *1.2.1* IGFBP-1, 3 and 5 are serine phosphorylated in their central domain (Coverley and Baxter, 1997). However this post-translational modification has been shown to have functional significance only in hIGFBP-1, which is phosporylated on serine residues at position 101 (70% of total phosphorylation), 169 (25%), 119 (5%) (Lee *et al.*, 1997). Phosphorylated hIGFBP-1 displays 4-6 fold higher affinity for IGF than the non phosphorylated isoform (Jones and Clemmons, 1995a). Rat IGFBP-1 is phosphorylated at Ser 107 (non homologous to human phosphorylated serines) and at Ser 132 (possibly corresponding to human residue Ser^{119}), but unlike the homologous human protein this post-translational modification does not affect the affinity for IGF-I (Peterkofsky *et* al., 1998).

The phosphorylation reaction occurs inside the cell, but the protein kinase responsible for IGFPB-1 postranslational modification has not been identified. Based on the sequences flanking the target serine residues, IGFBP-1 could be a suitable substrate for members of the casein kinase family and indeed casein kinase 2 was shown to phosphorylate IGFBP1 *in vitro* (Jones and Clemmons, 1995a). Rat IGFBP-1 phosphorylation is carried out in two different cellular sites, the *cis*-Golgi and the *trans* Golgi network, and together with casein kinase 2, the Golgi casein kinase has been implicated. (Peterkofsky *et al.*, 1998).

The observation that the ratio between phosphorylated and non phosphorylated hIGFBP-1 varies in different biological fluids and in physiological and pathological states suggested that this postranslational modification might have a regulatory role. It was reported that smooth muscle cells showed an increase IGF-I stimulated thymidine uptake when treated with non phosphorylated IGFBP-1 and a decrease when treated with the phosphorylated form (Busby *et al.*, 1988a; Jones and Clemmons, 1995a). Proliferating endometrial cells normally secrete non-phosphorylated IGFBP-1, but after medroxyprogesterone induction of luteinization, the cells produce phosphorylated IGFBP-1and undergo growth arrest (Frost and Tseng, 1991). IGF potentiating effects of the non phosphorylated form of IGFBP-1 have also been described in *in vivo* experiments, while the phosporylated isoform of IGFBP-1 did not display a potentiating action (Jyung *et al.*, 1994). It was hypothesized that phosphorylated IGFBP-1 could sequester IGF-I from the receptor because of its higher affinity and therefore inhibit the effects of the growth factor. Conversely the non phosphorylated IGFBP-1, which binds IGFs with lower affinity, could enhance IGF-I stimulated action perhaps by providing a reservoir of growth factors that can be released (Jones *et al.*, 1991).

However it should be borne in mind that the non phosphorylated isoform retains a high affinity for IGF with a K_a of 3.1-4.6 x10^9 (M^{-1}) (Koistinen *et al.*, 1993a and b), although it appears reduced in comparison to the phosphorylated form. In addition there are examples in the literature that contradict the generalized regulatory role attributed to phosphorylation. Both IGFBP-1 isoforms purified from amniotic fluid were able to block IGF-I binding to human fetal fibroblasts and to enhance IGF-mediated thymidine incorporation (Koistinen *et al.*, 1993a). In addition non phosphorylated IGFBP-1 was shown to inhibit IGF-stimulated cell growth *in vitro* (Burch *et al.*, 1990; Grellier *et al.*, 1996; Figueroa *et al.*, 1993) and *in vivo* (Motani *et al.*, 1995; Cox *et al.*, 1994). In conclusion the discrepancy in results derived from different experimental systems leaves the role of IGFBP-1 phosphorylation *in vivo* unclarified (Lee *et al.*, 1997). Perhaps the observation that it is the non-phosphorylated form that associates to cells may also be relevant for the interpretation of the function of phosphorylation on IGFBP-1 biology (Chan and Spencer, 1997).

hIGFBP-3 is secreted as phosphorylated protein and the modification principally occurs at serine 111 and 113 (Hoeck and Mukku, 1994). *In vitro* phosphorylation of IGFBP-3 can be obtained by protein kinase A and C and by casein kinase 2. The phosphorylation does not affect IGF-binding but it might affect its ability to associate to the cell membrane and to bind to ALS (acid labile subunit) (Coverley and Baxter, 1997).

Phosphorylation of IGFBP-5 is poorly reported.

1.2.1.2 GLYCOSYLATION

As shown in table 1.2.1, IGFBP-3 and -4 are N-glycosylated, while IGFBP5 and 6 are O-glycosylated.

IGFBP-3 is subjected to various degrees of glycosyl-substitution and contains three N-glycosylation sites, ^{89}NAS, ^{109} NAS, ^{172}NAS (Wood *et al.*, 1988) in the central region of the molecule. IGFBP-3 migration pattern in electrophoresis is represented as a doublet with an apparent Mr of 53-46kDa, compared to the theoretical Mr of 29kDa (Jones and Clemmons, 1995a). The biological significance of glycosylation is undetermined. The presence or absence of glycosyl residues in IGFBP-3 does not appear to affect IGF-binding nor the formation of the tertiary complex with IGF-I and ALS (Sommer *et al.*, 1993). Similarly both glycosylated and non glycosylated forms of IGFBP-3 can bind to heparin and be eluted from a heparin affinity column with 0.3-0.5M NaCl (Smith *et al.*, 1994).

Glycosylation of IGFBP-3 does not seem to be required for association to cell surfaces, as deletion of the central region of the molecule, which carries the 3 glycosylation sites, does not affect binding to cell membranes (Firth *et al.*, 1998).

IGFBP-4 is also N-glycosylated. In SDS gel electrophoresis under non reducing conditions, IGFBP-4 typically migrates as a double band with an apparent Mr of 28 and 24 kDa. Treatment with N-Glycanase reduces the intensity of the 28kDa band and at the same time increases the intensity of the 24 kDa band (Cheung *et al.*, 1991). Partial sequencing of the two bands show that they are indeed the same protein (Ceda *et al.*, 1991). The biological significance of this postranslational modification is unknown, but it is of interest to note that the 28kDa IGFBP-4 is slightly more resistant to proteolysis compared to the non-glycosylated form (Chernausek *et al.*, 1995).

IGFBP-5 is commonly described in literature as an O-glycosylated protein, but no precise details of this postranslational modification are available. In a recent report the comparison between theoretically calculated and spectrometrically analyzed mass of a C-terminal IGFBP-5 fragment shows a difference of about 1000Da (Standker *et al.*, 1998). The authors suggest that O-glycosylation at threonine ^{152}could account for this difference and that it might involve one hexose, one N' acetylhexosamine and 2 N'acetylneuraminic acid residues similarly to O-glycosylation described for IGFBP-6

Glycosylation of hIGFBP-6 has been the object of a detailed study recently published by Neumann *et al.*(1998). It was noted that IGFBP-6 displays a considerable heterogeneity of glycosylated isoforms. five different sites of glycosylation were identified on Ser or Thr residues of the central region of the IGFBP-6 molecule. Only Thr^{126}appeared to be glycosylated in all major isoforms, whereas substitutions at Ser^{144} Thr^{145} Thr^{146} Ser^{152}were variable. The number of carbohydrate residues was estimated between 8 and 16 in the major isoforms.

The significance of the glycosyl-substitution is unknown. It was noted that Ser and Thr residues involved in glycosylation of hIGFBP-6 were not conserved in mouse and rat sequence and that these proteins display a lesser extent of glycosylation. Though, similarly to IGFBP-4, the glycosylated form of IGFBP-6 appears to be less susceptible to chymotrypsin and trypsin digestion in comparison with the non glycosylated form, indicating that this post-translational modification might be biologically relevant (Neumann *et al.*, 1998).

1.2.1.3 RGD SEQUENCE

An arginine-glycine-aspartic acid (RGD) sequence is present in the C-terminal region of rat, cow and mouse IGFBP-1 (Brewer *et al.*, 1988; Hynes, 1992; Lee *et al.*, 1997) and in human, rat and chicken IGFBP-2 (Landale *et al.*,1995; Schoen *et al.*, 1995). The RGD sequence is a consensus recognition sequence for a family of cell membrane proteins, the integrin receptors. Integrins are involved in a multiplicity of cellular biological events among which the interaction between cells and extra cellular matrix (ECM) and cell migration are the best known. IGFBP-1 binds to $\alpha_5\beta_1$ integrin (fibronectin receptor) through its RGD sequence as first shown by the work of Jones *et al* (1993b). In order to prove IGFBP-1 binding to integrin, membrane proteins of CHO transfected cells expressing IGFBP-1 were iodinated and the cell lysates were applied to an IGFBP-1 affinity column and eluted with a competing RGD-containing peptide. Subsequently the eluted material was immune-precipitated with an antibody against the α_5 or β_1 integrin subunit and separated by electrophoresis. The direct autoradiography led to the identification of 2 single protein species with a Mr of 135 and 130 kDa (under reducing conditions). In contrast the use of an RGE containing peptide was unable to displace the CHO membrane proteins bound to the IGFBP-1 chromatographic column demonstrating that the RGD sequence of IGFBP-1was specifically involved in the interaction with the cell membrane. In addition the use antibodies to α_2, α_3, α_v subunits under identical condition to the anti α_5 antibody did not lead to the precipitation of any CHO membrane protein showing that IGFBP-1 binds specifically to the α_5 integrin subunit. Further support for these conclusions came from the demonstration that the anti α_5 antibody could co-precipitate IGFBP-1 bound to α_5 integrin on the cell membrane of the IGFBP-1-transfected CHO cells. In contrast when CHO cells were transfected with WGD-IGFBP-1 (which had the R of the RGD sequence substituted with W) the anti α_5 antibody was unable to co-immune precipitate WGD-IGFBP-1. Additional proof of the interaction of IGFBP-1 with $\alpha_5\beta_1$ integrin derives from the effect that the binding protein exerts in cell culture and *in vivo* experiments which will be reported in a later section..

Despite the presence of an RGD sequence in the C-terminal region of IGFBP-2, at the present time there are no reports showing IGFBP-2 binding to integrin receptors. Iodinated membrane proteins

from pSMC culture (pig smooth muscle cells expressing IGFBP-2) applied to an IGFBP-2 affinity column did not bind to the column. However the same cell lysate applied to an IGFBP-1 column led to the purification of $\alpha_5\beta_1$ integrin following elution of the column with RGD peptide (Gockerman *et al.*, 1995). Moreover in functional bioassays IGFBP-2 failed to stimulate cell migration of pSMC (Gockerman *et al.*, 1995) and wound healing (Galiano *et al.*, 1996) under the same experimental conditions which permitted the stimulating effect of IGFBP-1.

The presence of an RGD sequence is not *per se* a guarantee of heparin binding as several RGD containing proteins do not bind integrins (Gockerman *et al.*, 1995) and, in addition, integrins can bind proteins which lack an RGD sequence. Nevertheless, IGFBP-2 binding to integrins cannot be excluded as at least 6 different integrins recognize the RGD sequence and not all of them have been tested for binding IGFBP-2. Therefore it is possible that IGFBP-2 did not bind integrins expressed on pSMC cells ($\alpha_5\beta_1$ is the most abundant, although small amounts of $\alpha v\beta_3$, $\alpha_3\beta_1$ $\alpha_1\beta_1$ are also present) or that the expression of these integrins was quantitatively below the sensitivity of the detection system. Moreover the inability to detect any effect of IGFBP-2 on cell migration or wound healing does not exclude an efficient binding of IGFBP-2 and integrins. In fact the conditions and cell types used can affect the results of this type of bioassay even for known integrin –binding proteins. For instance Jackson and Yee (1995) were unable to demonstrate any binding of exogenous or endogenous IGFBP-1 to $\alpha_5\beta_1$ integrin expressed on the surface of MDA-MB-231(human breast tumour) and MG-63 (human osteosarcoma) cells. IGFBP-1 was also unable to compete cell attachment on fibronectin-coated plates. An indication of a potential functional role of IGFBP-2 RGD sequence was reported by Delhanty and Han (1993) who showed that wt IGFBP-2 potentiated IGF-II-stimulated mitogenesis in sheep choroid plexus cells whereas an RGE-IGFBP-2 mutant was ineffective. In conclusion, although the presence of an RGD sequence is neither necessary or sufficient for integrin binding the possibility that IGFBP-2 might bind to integrin needs further investigation.

1.2.1.4 HEPARIN-BINDING MOTIFS

Indications that the IGFBPs could bind to heparin were noted by researchers investigating the translocation of IGFs from the circulation to tissues. IGFs present in the plasma are mostly associated with IGFBP-3 and ALS (acid labile subunit) protein in a stable 150kDa complex which cannot leave the

circulation. It was observed that heparin could liberate IGF from the complex (Clemmons *et al.*, 1983). Heparin, produced by mast cells and stored in cytoplasmic granules, is released into the circulation during the inflammatory process . In these conditions heparin could function to liberate IGF from the 150 kDa complex and favour its delivery to the tissues. It was shown that IGFBPs bound to heparin and it was hypothesized that IGFBPs could also bind to GAGs (glycosaminoglycans) which are structurally related to heparin. GAGs are normal components of the cell surface and extracellular matrix and are believed to be involved in the translocation of IGFs to extravascular tissues. As it has been shown that IGFs can localize to specific cell types that do not express IGF mRNA but synthesize IGFBPs it was postulated that ECM/cell surface-associated IGFBPs could direct IGF to specific cell types within the tissue (Jones *et al.*, 1993a). While postulations about the biological meaning of the binding between IGFBPs and heparin were under investigation, this chemical property started to be exploited as an affinity chromatography step in the protein purification process for IGFBPs (Bar *et al.*, 1987).

Rat and human IGFBPs (1-6) (except rat and human IGFBP-4 and rat IGFBP-6) were found to have at least one heparin-binding consensus sequence (Hodgkinson *et al.*, 1994). Cardin and Weintraub (1989) identified, two heparin binding sequences: XBBXBX and XBBBXXBX ,where B represents any basic amino acid (mainly Arg and Lys) and X is unassigned.

hIGFBP-3 has 2 heparin binding motifs, one in the central region at position 148-153, , and one in the C-terminal region at position 219-226 (Firth *et al.*,1998). Both the central and C-terminal domain seem to account for IGFBP-3 ability to bind to heparin although to a different extent. In fact the deletion of the central domain of IGFBP-3 induced a reduction of heparin-binding, while the deletion of the C-terminus completely abolished the binding (Firth *et al.*, 1998). The heparin binding sequence in the C-terminal domain is included in a region formed by 10 basic amino acids between residues 215 and 232. An homologous basic amino acid-rich 201-218 sequence exists in IGFBP-5 and has been shown to be important for heparin/GAGs association. The 201-218 sequence of IGFBP-5 was able to compete with IGFBP-3 and IGFBP-5 for binding to fibroblast ECM indicating a possible role of these 18 residue homologous sequences in heparin/ECM binding (Parker *et al.*, 1996). In agreement with this hypothesis the substitution of the amino acids ^{228} KGRKR of IGFBP-3, included in the 215-232 sequence, with the

corresponding MDGEA sequence of IGFBP-1 caused a reduction in heparin affinity and abolished binding to the cell surface (Firth *et al.*, 1998).

hIGFBP-5 was first reported to have 3 heparin binding domains: two overlapping short heparin binding sequences (132-137 135-140) located in the central region of the molecule, while a third long-type sequence (205-212) heparin binding domain is in the C-terminal region of hIGFBP-5 (Hodgkinson *et al.*, 1994). In region 132-140 the two overlapping short consensus sequences form a VKKDRRKKLT sequence in which the BBXB heparin binding motif is in actual fact lost and it can therefore be considered as a basic amino acid rich region and not as an heparin binding sequence. An additional short heparin binding motif is identifiable at the 119-124 (PKHTRI) region of hIGFBP-5 (Parker *et al.*, 1996). The basic amino acid residues of the heparin binding sequence are believed to establish ionic bonds with the negative sulfate groups of heparin and possibly the same kind of interaction would also permit IGFBP-5 binding to the cell surface/ECM GAGs. In the attempt to test if the theoretically predicted heparin binding sequences were really responsible for IGFBP-5 binding to heparin and/or to GAGs several engineered IGFBP-5 constructs were prepared. An IGFBP-5 C-terminal 201-218 peptide, which contains 10 basic amino acids in a stretch of 18 residues and includes the long heparin binding sequence, was show to bind to heparin in ligand blot. A peptide with similar charge/mass ratio (containing 6 basic residues out of 11) spanning from residue 133 to 143 of IGFBP-5 central region performed less efficiently in heparin ligand blot (Andress, 1995). The $^{201\text{-}218}$ IGFBP-5 peptide was also demonstrated to compete IGFBP-3 or 5 binding to ECM although less efficiently than intact IGFBP-3 or 5. Instead the $^{131\text{-}141}$ IGFBP-5 peptide (7 basic amino acids out of 11), was considerably less effective in competing IGFBP-3 or 5 binding to fibroblast ECM. (Parker *et al.*, 1996). Thus it appears that heparin binding of IGFBP-5 depends in part on electrostatic interactions between basic amino acid of IGFBP-5 and heparin/GAG sulfated groups and in part on the specific position of the basic amino acids in the IGFBP sequence. As for IGFBP-3, the C-terminal region of IGFBP-5 seems to have a important role in heparin binding. The full length IGFBP-5 showed higher heparin affinity with respect to a corresponding C-terminally deleted molecule, as measured with $[H]^3$ heparin ligand blot or by the NaCl concentration required for the elution of the 2 molecules from heparin-Sepharose beads (Andress, 1995). However, despite the lower affinity for heparin,

the C-terminus truncated IGFBP-5 was able to bind to osteoblast monolayers where it exerted a mitogenic effect similar to intact IGFBP-5. (Andress and Birnbaum, 1992).

IGFBP-3 and 5 are the only IGFBPs that bind to fibroblast ECM. An 18 amino acid sequence rich in basic residues is present in IGFBP-5 (sequence 201-218), and in IGFBP-3 (215-232 sequence), but no equivalent can be found in IGFBP-1, 2 or 4. The above experiments indicated that these 18 amino acid sequences of IGFBP-3 and 5 were involved in heparin/GAGs binding. To further characterize the role of the 201-218 sequence of IGFBP-5 in heparin binding, the effect of the mutation of combinations of several basic residues within the 201-218 region was assayed. The basic residues ^{202}K, ^{206}K, ^{207}R, and ^{211}K were suggested to be the most important for binding to fibroblast ECM (Parker *et al.*, 1996) and in a later study the same authors (Parker *et al.*, 1998), concluded that the residues ^{214}R and ^{207}R are the two key residues for binding to the ECM of pSMC (pig smooth muscle cells). However the involvement of the heparin binding sequence in heparin and GAGs association was questioned by the finding that mutation of 2 basic residues within the sequence did not affect IGFBP-5 affinity for heparin whereas the mutation of other basic residues outwith the heparin binding motif, but inside the 201-218 sequence, did affect the binding (Arai *et al.*, 1996). In agreement with this result it has been reported that an IGFBP-5 derived peptide containing a short heparin binding sequence was unable to compete with IGFBP-5 for binding to pSMC ECM (Parker *et al.*, 1996).

Lastly heparin and ECM binding sites might not be identical as it was observed that a $^{131-141}$IGFBP-5 derived peptide containing 6 basic residues out of 11 was able to associate to ECM but not to heparin (Arai *et al.*1994a; Parker *et al.*, 1996).

As heparin inhibits IGFBP-5 binding to IGF-I (Arai *et al.*, 1994a) it was determined if the amino acid residues involved in heparin binding were also involved in IGF binding. First, IGFBP-5 analogs with mutated basic amino acid residues were tested for their affinity for IGF-I and showed no significant differences compared to wild type IGFBP-5 (Arai *et al.*, 1996). Next, the IGF-I affinity shift evident for wild type IGFBP-5 bound to heparin was evaluated and the impact of the basic amino acid mutations on this shift tested. Residues 211, 217 and 218, which do not significantly affect heparin binding nor IGF-binding, appeared to be involved in the shift of IGF affinity for the IGFBP-5-heparin complex (Arai *et al.*, 1996). Interestingly IGFBP-3 which has a sequence equivalent to 201-218 is not subjected to IGF affinity

shift after complexation with heparin. In addition, IGFBP-1, 2 or 4 binding to ^{125}I IGF-I was not affected by the addition of heparin (Arai *et al.*, 1994a).

Furthermore the IGFBP-5 201-218 sequence also seems to be involved in the recently described binding of plasma IGFBP-5 with PAI (plasminogen activator inhibitor) (Nam *et al.*, 1997) and with ALS (acid labile subunit) and similarly the homologous 215-232 sequence of IGFBP-3 is involved in the binding with ALS and plasminogen (Campbell *et al.*, 1998). The 210-218 IGFBP-5 and 215-232 IGFBP-3 sequences have also been recently shown to contain the NLS (nuclear localization sequence) a bipartite consensus motif that allows the intranuclear localization of both binding proteins (Schedlich *et al.*, 1998)

1.2.1.5 IGFBP PROTEOLYSIS

Proteolytic events have been described for all IGFBPs. Among many examples the proteolysis of circulating IGFBP-3 during pregnancy has been extensively studied.

IGFBP-3 is the most abundant IGF-binding protein in human plasma and it is easily detected in ligand blot as a typical 38.5-41.5kDa doublet band. However after the 6th week of pregnancy the IGFBP-3 doublet becomes progressively fainter until it disappears and it regains normal levels only 5 days post-partum (Giudice *et al.*, 1990). Similarly, in rat serum IGFBP-3 can hardly be detected after day 18 of pregnancy (Davenport *et al.*, 1992). It has also been demonstrated that the observed decline of IGFBP-3 in blood was due to a serum proteolytic activity which gave rise to IGFBP-3 fragments undetectable on ligand blot because of their decreased affinity for IGF. With IGF affinity cross-linking experiments it was shown that IGFBP-3 fragments retain some IGF binding capacity. The size of the fragments derived from the incubation of iodinated recombinant hIGFBP-3 with human term pregnancy serum was electrophoretically estimated at 29 and 22-17 kDa (Giudice *et al.*, 1990). The reduced affinity for IGFs that occurs for IGFBP-3 after proteolytic cleavage might have the function of improving the bioavailability of the growth factors for the increased pregnancy demand and fetal growth (Hossenlopp *et al.*, 1990).In addition Booth *et al.* (1996) propose a functional role for IGFBP-3 fragments on IGF passage through capillary walls.

The enzymes responsible for IGFBP-3 degradation in human and rat pregnancy serum, MMP-1 and MMP-2, are cation-dependent serine proteases belonging to the matrix metallo proteinase (MMP) family which includes MMP-1 (collagenase), MMP-2 (gelatinase A), MMP-3 (stromolysin 1) and MMP-9

(gelatinase B). These collagenases have a role in inflammatory process and metastasis invasion (Collet-Solberg and Cohen, 1996). Increased MMP activity during pregnancy might be due to a decline in the concentration of a tissue specific metallo proteinase inhibitors (TIMP). MMPs, unlike other serine proteases are not specific and degrade other IGFBPs in addition to IGFBP-3 (Clemmons, 1997). A small amount of proteolysed IGFBP-3 is present in normal adult rat (Lee and Rechler, 1995), but serum proteolytic activity can be considerably increased in stress conditions (late pregnancy, malnutrition, severe illness, malignancy and major surgery) (Jones and Clemmons, 1995a; Phillips *et al.*, 1998)

Plasmin and thrombin were also demonstrated to cleave IGFBP-3, but they are mainly located in vessels walls rather than in plasma (Clemmons, 1997; Zheng *et al.*, 1998a). Interestingly while heparin binding protects IGFBP3 from proteolysis by plasmin and pregnancy serum, it exerts a stimulatory action on thrombin activity. The modulation of the activity of different proteases might be another control point for IGF transport out of the circulation (Booth *et al.*, 1996).

Prostate specific antigen (PSA) is a kallikrein-like protease that is also able to degrade IGFBP-3 and 5, but not IGFBP-1, 2, 4 or 6 (Collett-Solberg and Cohen, 1996). This enzyme is found in seminal plasma and is present in high concentration in the blood of patients with disseminated prostate cancer (Clemmons, 1997).

γ-nerve growth factor is another kallikrein which is potently active on IGFBP-4 and 6 and consequently enhances IGF action showing an interaction between different growth factors (Collett-Solberg and Cohen, 1996)

In IGFBP-1 there is present one PEST (proline, glutamine, serine, threonine) domain which might increase the susceptibility of the protein to proteolysis. This sequence is only found in IGFBP-1, but other sequences in the central region of all IGFBPs are targets of protease action.

Pregnancy serum also degrades IGFBP-2, -4 and 5 (Davies *et al.*, 1991b; Claussen *et al.*, 1994). More than one proteolytic enzyme seems to be involved in this process as supported by the fact that the addition of specific inhibitors differentially affects the degradation of single IGFBP species. Recently a novel disintegrin-like MMP which cleaves IGFBP-3, -4 and -5 has been identified in pregnancy serum (Kubler *et al.*,1998). IGFBP proteolytic enzymes are also produced by cells in culture for example pSMC secrete 3 different proteases for IGFBP-2, IGFBP-4 and IGFBP-5 respectively (Rees *et al.*, 1998).

The action of proteases in cell culture can be modulated in various ways. Interestingly complexation of IGFBP-5 with IGFs protects it from enzymatic digestion (Collett-Solberg and Cohen, 1996; Arai *et al.*, 1994b; Zheng *et al.* 1998a) whereas IGFBP-4 proteolysis occurs at a very low rate in conditioned medium of human decidua and rat neuroblastoma cells (Myers *et al.*, 1993, Chernausek *et al.*, 1995) unless IGF is added. A change in conformation due to IGF binding might be responsible for both these opposing effects. IGFBP-5 is also protected by proteolysis when it is associated to the ECM (Clemmons, 1997; Nam *et al.*, 1997).

Cathepsin D is also able to proteolyse IGFBP-1 through 5, but its physiological significance is uncertain. In fact cathepsins are a family of lysosomal enzymes and although they can be found in the conditioned medium of prostate and breast cancer cells, they are active only at a pH ranging between 4 and 5.5 (Collett-Solberg and Cohen, 1996)

1.2.1.6 AFFINITY FOR IGFs

A large amount of data has been collected on the affinity of each IGFBP for IGF-I or –II and these data are summarized in table *1.1.2*

The general consensus is that IGFBP-1 and 4, have equal affinity for IGF-I or -II, while for IGFBP 2, 3, 5, the preferential binding to IGF-II in comparison to IGF-I is variously reported. Indisputable is IGFBP-6 higher affinity (20-100fold) for IGF-II (Drop *et al.*, 1992; Oh *et al.*, 1993; Roghani *et al.*, 1991).

Many studies have investigated the IGF-binding domain on IGFBPs although the absence of information on IGFBP tertiary structure rendered these investigations very difficult. The possibility of defining the IGF binding site by using mutated or truncated IGFBPs was limited by the fact that loss of IGF-binding ability could either be due to the effect of the introduced modification or simply a consequence of major changes in the tertiary structure of the IGFBP molecule. During recent years a conspicuous amount of data have been accumulated alternatively supporting the hypothesis that the IGF-binding domain was located either in the N-terminus or in the C-terminus of the IGFBP molecule.

In favour of the primary role of the N-terminal domain of IGFBP there was the observation that the N-terminus retained IGF-binding capacity when isolated from the rest of the molecule, while deletion or alteration of the N-terminal domain caused complete loss of IGF-binding. Similar results were obtained

by different researchers with different IGFBPs. An IGFBP-1 molecule deleted of the first 60 N-terminal amino acids was unable to bind IGF-I in ligand blot (Brinkman *et al.*, 1991a). Mutation of IGFBP-1 Cys residues at position 16 or 35 into serines (Powell *et al.*, 1989) or 38 into Tyr (Brinkman *et al.*, 1991a), caused a complete loss of IGF binding. This is likely to be due to a major change in the molecular integrity rather than to the loss of a residue essential for IGF binding. In contrast point mutations introduced in the N-terminus of the molecule or even the mutation of Cys 34 to Tyr did not affect IGF binding, suggesting that Cys34 may not be involved in maintaining IGFBP-1 tertiary structure (Brinkman *et al.*, 1991a). The comparison of proteolytic fragments from the digestion of IGFBP-3 with plasmin, thrombin or pregnancy serum showed that the most of the N-terminus containing peptides retained IGF binding affinity in ligand blot, while only a few C-terminal fragments, starting approximately around residue 150, displayed IGF binding (Booth *et al.*, 1996). Accordingly a naturally occurring 30 kDa N-terminal proteolytic fragment (1-160) of IGFBP-3 was capable of IGF binding, but a smaller 16 kDa (1-95) N-terminal fragment was not (Mohseni-Zadeh and Binoux, 1997b). In affinity cross-linking experiments it was found that the N-terminal or the N-terminal linked to the central region of IGFBP-3 maintained IGF-binding affinity (Sommer *et al.*, 1991; Hashimoto *et al.*, 1997; Firth *et al.*, 1998). The affinity of the N-terminus (1-93) or the N-terminus+central region (1-186) was also quantified in a competitive solid phase assay and determined to be respectively 4% and 12.5% of wtIGFBP-3 IGF-binding ability (Hashimoto *et al.*, 1997). The discrepancy of the IGF-binding ability of small fragments might depend on the detection method. In general the reduced affinity of the fragments becomes undetectable in ligand blot, while it can be revealed with ^{125}IIGF-I or II cross-linking techniques. Hobba *et al.*(1996) reported that iodination of Tyr ^{60} residue of bIGFBP-2 decreased IGF-affinity. Two years later the same authors confirmed the importance of Tyr^{60} showing that this Tyr was the only residue, within the Val^{59} and Arg ^{63} region of bIGFBP-2, whose mutation caused a reduction in affinity for IGF-I and II (Hobba *et al.*, 1998). Further support to the primary role played by the N-terminal domain in IGF binding is provided by Kalus *et al.*(1998). The authors found that of three proteolytic fragments corresponding to the C-terminus, the central region and the N-terminus of hIGBBP-5, only the latter retained the ability to bind to IGF-I or -II. In particular the IGF binding domain seems to be confined to an Ala^{40}-Ile^{92} mini-fragment of IGFBP-5 which appears to be sufficient to start the IGF-binding process with a very high

association constant. However this mini-IGFBP-5 fragment dissociates from IGF very quickly, so that its affinity is 10-200 fold reduced compared with the full length IGFBP-5. Therefore the higher affinity of the full length IGFBP-5 must be due to some additional binding interactions established by the central and/or the C-terminal regions that may have the effect of stabilizing the IGF-IGFBP-5 complex. Similar conclusions were drawn by Qin *et al.* (1998) who expressed isolated domains of IGFBP-4 as GST fusion proteins and found that i) C-terminal domain was unable to bind IGFs, ii) the deletion of the central region (which in IGFBP-4 contains two additional Cys residues) did not affect the affinity, iii) N-terminus of IGFBP-4 truncated at various length retained binding capacity but with a substantially lower affinity compared to the full length protein

Other studies supported the C-terminal domain as the candidate for the location of the IGF binding domain. A 21kDa fragment corresponding to 148-270 of rIGFBP-2 retained a low affinity for IGF-I or-II, (Wang *et al.*, 1988). An IGFBP-1 molecule lacking the 20 C-terminal amino acids was unable to bind IGF in ligand blot. However point mutation introduced in the C-terminus of IGFBP-1 did not affect IGF binding and only the substitution of Cys 226 with a Tyr or the introduction of an amino acid negatively charged in the vicinity led to formation of dimers and loss of IGF binding (Brinkman *et al.*, 1991b). A 14kDa carboxyl-terminal fragment of IGFBP-2 isolated from human milk retained considerable affinity for IGF-II in RIA assay (Ho and Baxter, 1997a).

In conclusion the cooperation of both the N-and C-terminal domains of IGFBPs appears to be needed for IGF binding. Moreover it should be pointed out that despite the high homology between IGFBPs, each IGFBP species shows different affinity for IGF-I and II. Therefore it is possible that in each IGFBP the relative contribution of a defined part of the molecule might vary. Furthermore, the observation that truncation or mutation of the IGFBP molecule differentially affected the affinity for IGF-I or -II gave rise to the hypothesis that binding sites for the two peptides might be overlapping but not identical.
Iodination of bIGFBP-2 Tyr^{60} residue (Hobba *et al.*, 1996) or its mutation to Phe caused 8 fold reduction in affinity for IGF-I and 4 fold reduced affinity for IGF-II (Hobba *et al.*, 1998). A bIGFBP-2 molecule whose 62 C-terminal residues were deleted had a general reduction in affinity for both IGFs, but the loss of affinity for IGF-II was more dramatic (Forbes *et al.*, 1998). In cross-linking affinity labeling assays an IGFBP-3 lacking the C-terminal domain showed a big reduction in affinity for both IGFs but IGF-II

binding was most compromised (40 fold reduction, compared to 20 fold reduction in affinity for IGF-I). Moreover while the mutation of ^{253}KED sequence in the central region of IGFBP-3 into RGD affected the affinity for both IGFs (a 4-6 fold reduction), the substitution of ^{228}KGRKR sequence with MDGEA in C-terminal region of IGFBP-3 caused nearly no changes in IGF-I affinity, but a 3 fold reduction for IGF-II (Firth *et al.*, 1998). Similarly a 14 kDa C-term fragment of hIGFBP-2 had only 10 fold lower affinity for IGF-II compared with wt IGFBP-2, while its binding capacity for IGF-I was much more seriously compromised (Ho and Baxter, 1997a).

Finally some reports have described equivalent effects on IGF-I and IGF-II affinity following IGFBP modifications. For example a 21kDa fragment corresponding to the 148-270 C-terminus of rIGFBP-2 bound IGF-I or-II equivalently, although with low affinity (Wang *et al.*, 1988). Similarly Qin *et al.*(1998) found that the N-terminal fragments of IGFBP-4 had a similarly decreased affinity for ^{125}I IGF-I or -II in ligand blots.

In summary, from the comparison of studies undertaken by different laboratories, it appears that in some IGFBP species, the C-terminal region might contain determinants which are specifically important for IGF-II binding, while the N-terminal region might be involved in more fundamental aspects of the binding mechanism which applies to both IGF-I and -II.

1.2.2.IGFBP FUNCTIONS

1.2.2.a In vivo

1.2.2.a.1 Serum transport and storage of circulating IGF

Ternary complex Perhaps the most intensively studied function of IGFBPs is as a carrier of IGFs. Just after IGFs were discovered as a biological mediator of GH action, it became clear that the growth factors were not present in plasma as free form. 90-96% of IGF-I and II are bound in a 150kDa complex together with IGFBP-3, a 46-53 kDa N-glycosylated acid stable protein, and to ALS, an acid labile subunit of the complex (Clemmons, 1997). ALS is a glycoprotein with an apparent Mr of 84-86 kDa and a characteristic leucine-rich domain which is believed to facilitate protein-protein interaction (Leong *et al.*,

1992). Liver is the main source of plasma IGF, IGFBP-3 and ALS. The proteins are released into the circulation where i) IGF combines with IGFBP-3 in a dimer complex and ii) subsequently binds to the ALS subunit forming a stable 150 kDa ternary complex (Jones and Clemmons, 1995a). ALS is present in excess in the blood (290nM), while IGFBP-3 is totally saturated with IGF so that the growth factors and the binding protein have almost equimolar concentration in the circulation.(120-150nM) (Phillips *et al.*, 1998). The assumption that virtually all plasma IGF and IGFBP-3 are complexed with ALS in the 150kDa complex, has now been challenged. Collet-Solberg *et al.* (1998) described three blood association proteins (APs), with a Mr of 70, 100, 150kDa, able to bind IGFBP-3 and they propose that part of IGFBP-3 in the serum is associated to these APs and part with ALS. Campbell *et al.* (1998) suggest an intriguing hypothesis based on the observation that IGFBP-3 binds to plasminogen and that the complex, present in normal plasma, is able to bind IGF-I with high affinity (K_d 0.47 nM). The theory that all IGFBP-3 present in the plasma is bound to ALS (88kDa) and IGF is based on gel filtration studies which simply show that plasma IGFBP-3 is engaged in a 150 kDa complex. However as plasminogen (97-92 kDa) has a Mr similar to ALS, it is possible that part of the 150 kDa is in actual fact formed by IGFBP-3, IGF and plasminogen. On the other hand an IGFBP-3-ALS-plasminogen trimeric complex would not form as both ALS and plasminogen interact with the 215-232 heparin binding sequence in the C-terminus of IGFBP-3.

The belief that IGFBP-3 could not form a binary complex with ALS has been questioned by a number of studies proving that binding between IGFBP-3 and ALS in the absence of IGFs could indeed be detected using several techniques (Lee and Rechler, 1995; Hashimoto *et al.*, 1997; Collett-Solberg *et al.*, 1998). Moreover the 150 kDa complex was also found in ovary follicular fluid demonstrating that it is not characteristic of the plasma only (Cwyfan Hughes *et al.*, 1997), neither is its formation an exclusive property of IGFBP-3, as IGFBP-5 has recently been reported to form a 150 kDa complex with ALS and IGF in serum (Twigg and Baxter, 1998).

The 150kDa complex represents a storage form of IGF-I/II in the body. The renal clearance of the 150kDa complex is limited and extravascular passage is impeded resulting in an increased plasma half life of 12-15 hours compared to free IGFs (10 min) and free IGFBP-3 (1-2 hours) (Jones and Clemmons, 1995a). It is of fundamental importance that the relative concentration of all components of the ternary complex is maintained in equilibrium as indicated by the fact that hepatic production of these three

components is subjected to positive GH regulation. It is worthwhile to note that an increased availability of free IGFs, e.g. after an injection of IGF peptide, would cause an acute hypoglycemic effect if it was not buffered by IGFBP (Collett-Solberg and Cohen, 1996). Any change in relative concentration of one of the components of the complex is followed by an adjustment of the concentration of the other components. For instance any rise in plasma IGFs stimulates an increase of IGFBP-3, while ALS is available in excess. Subsequently, in order to maintain constant the concentration of 150kDa complex, IGF, which negatively regulates GH secretion, indirectly inhibits ALS secretion. Equilibrium is then restored because a larger proportion of binary complex IGF-IGFBP-3 is formed and quickly cleared from the circulation (Clemmons, 1997). The physiological function of this circulating store of IGF is not fully understood but there are indications which suggest that it might represent a body reservoir to be used in catabolic conditions. In fact it has been observed that in stress states (eg late pregnancy-Hossenlopp *et al.*, 1990; Giudice *et al.*, 1990; Lasarre and Binoux, 1994; diabetes- Bang *et al.*, 1994; Bereket *et al.*, 1995; malnutrition-Pucilowska *et al.*, 1993, severe illness- Davies *et al.*, 1991a; malignancy- Muller *et al.*,1993 and major surgery Davenport *et al.*, 1992) IGFBP-3 is specifically cleaved by serum proteases into a 30 kDa fragment still able to form the ternary complex but with a 20-30 fold decreased affinity for IGF (Binoux *et al.*, 1991). The growth factors can then be easily released from the ternary complex and equilibrate with lower Mr IGFBPs or with the extra vascular tissues (Martin and Baxter, 1992).

IGF-IGFBP binary complex The small portion of IGFs, less than 10 %, which is not included in the trimeric complex forms a 40-50kDa dimeric complex, while only 0.4% is in a free form (Chan and Spencer, 1997). However, although in normal conditions most of IGFs are bound to the 150kDa complex, in pathological conditions the concentration of the trimeric complex can decrease to favor smaller Mr complexes between IGF and different classes of IGFBPs (Hardouin *et al.*, 1989; Zapf *et al.*, 1990; Martin and Baxter, 1992). IGFBP-1, -2 and, to lesser extent, IGFPB-4 are present in plasma in significant concentration, while IGFBP-5 and 6 levels are very low and their role in the physiological regulation of circulating IGF is unclear (Clemmons, 1997). Unlike the 150kDa complex, the binary complex of IGFs with IGFBP-1, -2 or -4 can cross capillary barriers, and may be facilitating the delivery of the growth factors to peripheral tissues (Bar *et al.*, 1990a). IGFBP1 and 2 can actively influence IGF bioavailability especially in pathological conditions. Both proteins are present in the plasma in unsaturated form

(McCusker et al., 1988) and although they are 10 times less concentrated (2-15 nM –Murphy, 1998) than IGFBP-3 and have 5-10 fold lower affinity for IGFs, they are available to bind any IGFs liberated from the 150 kDa complex. The movement of IGF from the high to the low Mr complex is not fully elucidated and no answers have been found to explain why some IGF circulates free despite the presence of unsaturated IGFBPs (Murphy, 1998).

IGFBP-1 IGFBP-1 is metabolically more responsive than IGFBP-3 with only a 14 min half-life in the circulation (Lee *et al.*, 1997). The IGF-IGFBP-1 complex is not comparable to the 150kDa trimeric complex and its rapid and dynamic regulation is exploited to modulate IGF bioavailability in specific physiological or pathological states. Liver is the main source of plasma IGFBP-1 in males and non pregnant females. Liver production of IGFBP-1 is mainly under insulin negative regulation. Insulin causes a fall in IGFBP-1 concentration within 15min. of administration which, together with the lack of effect of cyclohexamide (a translation inhibitor) treatment suggests that no transcription factor synthesis is required for the inhibition to occur (Phillips *et al.*, 1998). Moreover insulin seems to be able to stimulate IGFBP-1 capillary transport, further contributing to decreased IGFBP-1 plasma concentration (Bar *et al.*, 1990b). In a parallel fashion insulin up-regulates IGFBP-3 and increases IGF-I concentration (Phillips *et al.*, 1998) Thus in normal conditions, GH positively regulates the concentration of plasma 150 kDa complex either by acting directly or controlling insulin levels, which also suppress IGFBP-1 concentration. As the IGFBP-1 gene is subjected to negative insulin regulation through an IRE (insulin responsive element) region located at the transcription start site (Lee *et al.*, 1997), IGFBP-1 protein levels are readily influenced by changes in nutritional status. The postprandial rise in insulin level causes 4-5 fold decrease in IGFBP-1 concentration. Conversely fasting for 12-16 hours stimulates a 4-6 fold increase in IGFBP-1 concentration, due to suppression of insulin levels (Busby *et al.*, 1988b). A decrease of plasma levels of 150kDa complex and an increase in IGFBP-1 is typical of diabetes and GH deficiency (Graubert *et al.*, 1991).

IGFBP-2. IGFBP-2 is the most abundant IGFBP in serum after IGFBP-3. Like IGFBP-1, IGFBP-2 in plasma is present in unsaturated form, but it is less responsive than IGFBP-1 to metabolic changes and it has a longer half life (90min) (Jones and Clemmons, 1995a). Nutrition is a weak regulator of IGFBP-2 concentration. Severe protein deprivation can increase (30-40%) the IGFBP-2 level , but only,

after 5-6 days (Clemmons *et al.*, 1991; Straus and Takemoto, 1990). GH directly down-regulates the concentration of the binding protein, whereas IGF-II potently increases IGFBP-2 levels. A decrease in the 150 kDa complex concentration and a compensatory increased level of IGFBP-2, is the typical plasma pattern of patients affected by non-islet cell tumor hypoglycemia (LeRoith, 1996). This tumor produces an high level of big IGF-II, an IGF-II variant with C-terminal 21 amino acid extension represented by uncleaved residues of the E domain. The origin of hypoglycemia associated with this tumor is not clear as IGF-II concentration is not always high in these cases. It has been proposed that an impaired formation of the 150 kDa complex might be the cause of hypoglycemia (Hizuka *et al.*, 1998). In this type of tumor GH is usually at low level (because IGF-II inhibits pituitary GH secretion) and as consequence IGF-I, IGFBP-3 and ALS are low and this could be limiting for the formation of the 150kDa complex.

The relative abundance of the IGFBPs does not only change in pathological conditions but also in different physiological states.

1.2.2.a.2 IGFBPs in foetus and pregnancy

In human foetal serum IGF-II, IGFBP-2 and IGFBP-1 concentrations are high, whereas IGF-I and IGFBP-3 are low. Under GH stimulation IGF-I starts to increase around birth as does IGFBP-3 whose concentration is very low in young children, then it increases and reaches its peak at mid puberty and only starts to decline after age 30. Conversely post natal levels of IGF-II, IGFBP 1 and 2 decline (LeRoith, 1996, Clemmons, 1997, Lee *et al.*, 1997).

During pregnancy the maternal endometrium secretes into the amniotic fluid an extremely high quantity of IGFBP-1which causes IGFBP-1 levels in maternal plasma to double, although they are still one order of magnitude lower then in the amniotic fluid. Foetal plasma concentration of IGFBP-1 is between the levels in amniotic fluid and maternal plasma, and it starts to be highly expressed in fetal hepatocytes only in the perinatal period. The localized, time-regulated and substantial changes in the expression of IGFBP-1 during pregnancy suggests a specific role for this protein. One hypothesis is that IGFBP-1 is involved in the process of trophoblast invasion of maternal endometrium. This occurs in a series of subsequent steps: i) cell attachment, ii) local matrix proteolysis, iii) cell migration and cessation of these activities. It has been observed that high levels of IGF-II are expressed at the invading front resulting in a stimulation of trophoblast cell proliferation and migration. Thus IGFBP-1 could act by

inhibiting IGF-II interaction with the receptor and therefore reduce trophoblast invasion. On the other hand IGFBP-1, through its interaction with cell membrane $\alpha_5\beta_1$ integrin, has been shown to stimulate of CHO cells and long-term passaged cytotrophoblast migration in culture conditions (Jones et al., 1993b; Irving and Lala, 1995). It is likely that the combination of the two opposite effects of IGFBP-1 on cell migration would result in a final inhibiting effect *in vivo* (Lee *et al.*, 1997). Recently, Irwin and Giudice, showed that freshly isolated cytotrophoblasts were inhibited in the invasion of human endometrial cells (decidualized *in vitro*) when IGFBP-1was expressed, but not when IGFBP-1 expression was suppressed by insulin. The authors suggest that in this case IGFBP-1, by binding to the integrin receptor, restrains trophoblast invasiveness acting in a fibronectin-like fashion (Irwin and Giudice, 1998).

1.2.2.a.3 Wound healing

In a rabbit ear dermal ulcer model IGFBP-1 has been described to promote wound healing when locally applied in combination with IGF. This is another effect of IGFBP-1 which occurs through the interaction of its RGD sequence and integrin receptor. IGFBP-1 or IGF alone were ineffective as were the combination of IGF-I-IGFBP-2-, IGF-I-WGD-IGFBP-1 (an IGFBP-1 analog with a point mutation of the first amino acid of the RGD sequence) and Des(1-3) IGF-I-IGFBP-1, demonstrating that binding between the growth factor and the binding protein-1 and the integrity of IGFBP-1 RGD sequence were essential for this effect (Galiano *et al.*, 1996). A role for IGFBP-1 in wound healing has also been studied in humans, rats and in diabetic rabbits (Kratz *et al.*, 1994; Jyung *et al.*, 1994; Tsuboi *et al.*, 1995).

1.2.2.a.4.Foetal development

The deletion of IGF-I and II mouse genes by homologous recombination produced animals of normal features, but reduced in size (DeChiara *et al.*, 1990; Powell-Braxton *et al.*, 1993) indicating the involvement of the two peptides in foetal development. A role for IGFBPs in foetal development was also postulated, acting either through the modulation of IGF action or in an IGF-independent fashion. IGFBP expression in rat and mouse embryos has been determined and a specific spatio-temporal expression pattern was observed (Baker *et al.*, 1993; Powell-Braxton *et al.*, 1993; Cerro *et al.*, 1993; Green *et al.*, 1994; Schuller *et al.*, 1993; Streck *et al.*, 1992; Wood *et al.*, 1992). In particular, IGFBP-2 and-5 expression appears to be localized in the same or adjacent tissues (Green *et al.*, 1994; Schuller *et al.*, 1993)

suggesting a complementary role of these two binding proteins in specific tissues (Allan *et al.*, 1999). The targeted disruption of the IGFBP-2 gene in mice did not produce any apparent phenotypical alteration (Wood *et al.*, 1993). This result, while suggesting a functional redundancy between the 6 IGFPBs, does not exclude a specific role for each IGF-binding protein in the development of normal embryos.

1.2.2.a.5 delivery to specific tissue

It is believed that IGFBPs can play a role in IGF transcapillary transport. Low Mr IGF-IGFBP complexes can cross capillary barriers (Jones and Clemmons, 1995a) and a specific role for endothelium associated IGFBP-3 fragments has been proposed (Booth *et al.*, 1996). IGFBP-1 has been identified in peritoneal fluid whose composition is believed to be similar to interstitial fluid. It is not known if the passage of IGFBP-1 to the extravascular compartment occurs by passive leakage from the blood or if it involves an active/regulated transport which would further support the hypothesized role of IGFBP-1 in the interstitial space (Lee *et al.*, 1997). During the acute phase response in CNS injury, IGF-II is rapidly mobilized from the central nervous fluid to the damaged neural parenchyma, in the absence of increased local mRNA expression. It has been proposed that IGFBP-2, which is the most abundant IGFBP species in the CNS and whose local expression also increases, might play a specific role in IGF-II transportation in the wounded site (Walter *et al.*, 1999; Walter *et al.*, 1997).

1.2.2.a.6 organ specific functions.

IGF growth promoting and metabolic action is widely expressed throughout the organism, but it might play a specific role in defined organs where the modulation by IGFBPs will significantly influence IGFs biological effects. However the widely spread tissue production of the 6 binding proteins, the contemporary presence of growth factors and proteases make the investigation of IGFBP effects on a single organ very complex. For this reason much of the knowledge on the biology of IGFBPs derives from studies carried out in suitable *vitro* models.

1.2.2.b in vitro

Both stimulating and inhibiting effects on IGF action have been attributed to IGFBPs in different cell culture systems. It is generally believed that soluble IGFBPs have an inhibiting effect on IGF action as

they display high affinity for IGFs and prevent the growth factors from interacting with IGF-IR. In contrast, most of the IGF-potentiating effects attributed to IGFBPs refer to cell surface or to ECM-associated IGFBPs. Although exceptions to this generalization have been reported, it is becoming apparent that the soluble and immobilized forms of the same IGFBP can have quite different biological actions.

The nature of the interaction between IGFBPs and the cell surface or the ECM has not been fully elucidated. As mentioned in the previous sections the presence of the RGD sequence in IGFBP-1 and 2 suggests a possible interaction of these IGFBPs with integrin receptors. Alternatively (or in addition to the RGD sequence) IGFBPs might bind to the ECM or the cell surface through a heparin binding motif (present in all IGFBPs) or through an 18 amino acid region rich in basic residues (only in IGFBP-3 and 5). It has been proposed that in this case the binding would occur via GAG components of the ECM or the cell surface. However the molecular features of this interaction are not completely clarified. It remains to be explained why, although all IGFBPs display at least a heparin binding sequence, different IGFBPs show a preferential binding to the cell surface or the ECM or why some IGFBPs do not seem to bind either the cell surface or the ECM. The understanding of the mechanisms involved in this association would be useful for a better interpretation of the contradictory effects displayed by the IGFBPs in a variety of biological assays

1.2.2.b.1 IGFBP-1

Either IGF- inhibiting or potentiating effects have been attributed to IGFBP-1. as reviewed by Jones and Clemmons (1995a); LeRoith (1996);Collett-Solberg and Cohen (1996), and Lee *et al.* (1997).

Inhibition of IGF action.

In general if IGFBP-1 is phosphorylated or present in at least 4:1 excess of IGFs, it exerts an inhibitory effect on IGF action (Clemmons, 1997). Phosphorylated IGFBP-1, which has higher affinity for IGF-I, decreased IGF-I-stimulated DNA synthesis in human fibroblasts, but after dephosphorylation IGFBP-1 potentiated IGF action (Busby *et al.*, 1988a; Jones and Clemmons, 1995a). If a high enough molar excess of protein is used, there is no requirement for IGFBP-1 to be phosphorylated in order to exert inhibition on IGFs. In MCF7 cells IGFBP-1 inhibits IGF-I stimulated growth, preventing the phopshorylation of the IGF-IR by IGF-I (Yee *et al.*, 1994). In the same breast cancer cell line IGFBP-1

also inhibits estrogen or serum-stimulated cell growth (Figueroa *et al.*, 1993). When added in excess, IGFBP-1 has also been reported to inhibit: i) AIB uptake in JEG-3 choriocarcinoma cells (Ritvos *et al.*, 1988), ii) ^{3}[H] thymidine incorporation in pSMC (Busby *et al.*, 1988a) and in MG63 osteosarcoma cells (Campbell and Novak, 1991) and iii) glucose uptake in 3T3/Balb fibroblasts (Okajima *et al.*, 1993).

Potentiation of IGF action.

In contrast when the non-phosphorylated isoform of IGFBP-1 is used, or when the binding protein is added at low concentrations, an IGF potentiation is generally obtained. IGFBP-1 enhanced IGF-I, but not IGF-II, stimulated proliferation in human keratinocytes and skin fibroblasts (Kratz *et al.*, 1992) and increased DNA synthesis in human, mouse and chick embryo fibroblasts (Elgin *et al.*, 1987). In contrast to the above mentioned inhibitory effect of IGFBP-1 in pSMC, this binding protein was reported to increase 4.8-fold the IGF-I-stimulated ^{3}[H]thymidine uptake, while IGFBP-2 only increased IGF action 2.2-fold under the same conditions (Bourner *et al.*, 1992).

IGFBP-1 interaction with integrins.

The interaction of the C-terminal RGD sequence of IGFBP-1 with the cellular $\alpha_5\beta_1$ integrin (fibronectin receptor) on the cell surface has been described (Jones *et al.*, 1993b). Post-translational modifications might influence this binding as it has been reported that only the phosphorylated form of IGFBP-1 can associate to the cell surface (Chan and Spencer, 1997).

IGFBP-1 stimulates cell migration in CHO cells (Jones *et al.*, 1993b) and in pSMC cells seeded on fibronectin coated plates (Gockerman *et al.*, 1995).

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

IGF-I and II alone are also potent stimulators of pSMC cell migration on fibronectin coated plates. In this case IGFBP-1 exerts an inhibiting effect on IGF-stimulated migration by antagonizing the binding of the growth factors to IGF-IR and consistently it fails to affect des(1-3)IGF-I action in this assay system. (Gockerman *et al.*, 1995).

Cell migration is a complex phenomenon resulting from the interaction between several growth factors, the cells and the extracellular matrix (Gockerman *et al.*, 1995). Therefore a combination of factors, including the integrin type expressed on the cell surface and the cellular substratum, can affect the ability of a molecule to stimulate cell migration.

Thus on vitronectin coated plates IGF-I also promotes pSMC cell migration. but in this case the contemporary stimulation of the IGF-IR by IGF-I and the ligand occupancy of $\alpha_v\beta_3$ integrin (vitronectin receptor) seem to be necessary. The prevention of these events by the addition of IGFBP-1in the former case or $\alpha_v\beta_3$ integrin competitors (such as echistatin and kistrin),in the latter case, inhibits IGF-I-stimulated cell migration (Jones *et al.*, 1995b; Jones *et al.*, 1996).

Integrins that have been considered principally as adhesion molecules, are now increasingly reported to be involved in cellular signaling events. Integrin α subunit is responsible for ligand specificity, while the β subunit determines the signal specificity. This means that the stimulation by different ligand molecules can lead to the same cellular response. Integrin β subunit does not have tyrosine kinase activity, but (at least for $\beta_1$and β_3) can associate with $pp125^{FAK}$ (focal adhesion kinase). This kinase is activated by phosphorylation and concentrates around the focal adhesion area, which represents the integrin-cytoskeletal transmembrane linkage. Other proteins have been reported to become phosphorylated in response to integrin signalling such as IRS-I, Grb2, and MAP kinases suggesting a convergence between integrin and IGF-IR signalling (Vuori and Ruoslahti, 1994; Schlaepfer *et al.*, 1994; Chen *et al.*, 1994; Morino *et al.*, 1995). Importantly, treatment with 100nM insulin, a dose that would be able to stimulate the IGF-IR, stimulated phosphorylated IRS-I to associate to the $\alpha_V\beta_3$ receptor (Vuori and Ruoslahti, 1994)

IGFBP-2

A detailed description of IGFBP-2 interaction with the cell membrane/ECM and its biological function in cell culture systems will be described in the last paragraph of this literature review as it relates to the principle investigation of the current thesis.

1.2.2.b.2.IGFBP-3

IGFBP-3 biological functions include an inhibitory and a potentiating effect of IGF action, as well as an IGF independent effect. Data reported in the literature are often contradictory and the attempt to deduce some general properties of IGFBP-3 behavior in the different cell systems is difficult. However it seems clear that IGFBP-3 interaction with the cell surface/or ECM, and possibly the different methods by which this takes place, can largely influence the IGFBP-3 effect. In the past five years IGFBP association with the cell membrane or with the ECM has been extensively investigated. IGFBP-3 and 5 were the most thoroughly studied binding proteins with this respect for two main reasons: i) first because in various cell cultures a significant part of IGFBP-3 and 5 appears to be bound to the cell surface/ECM, ii) second, and probably more important, because the study of cell surface/ECM association represents a new promising approach for understanding the mechanisms by which IGFBP-3 and 5 can either potentiate IGF action or act in an IGF-independent fashion. The molecular aspects of IGFBP cell surface/ECM-association have not been fully identified, however the two most popular theories propose either GAGs or protein molecules as cell membrane mediators of IGFBP binding.

A summary of some of the studies in support or in contradiction of the role played by GAGs in IGFBP-3 association to the cell membrane will be introduced before the description of the biological effects of IGFBP-3. The interaction of IGFBP-3 with a putative membrane IGFBP-3 receptor protein and IGFBP-3 nuclear translocation will be mentioned in the section dedicated to IGFBP-3 (IGF-independent) effects.

It was shown that heparin could release cell-associated IGFBPs into the culture medium (Martin *et al.*, 1992b). Heparin is very similar to heparan sulfate, one of the GAGs present on the cell membrane and the ECM. Both are polymers formed by highly sulfated iduronic acid-N-acetylglucosamine disaccharide repeat units. Thus it was hypothesized that the mechanism by which heparin displaced cell-associated IGFBP was a specific competition between heparin and the cell membrane heparan sulfate. It was subsequently shown that heparin displacement of fibroblast or rat glioma cell-associated IGFBP-3 is

likely to be due to the direct binding between heparin and IGFBP-3 which possibly masks the binding sites necessary for IGFBP-3 association to the cell surface (Yang *et al.*, 1996). Oh *et al.*(1992) showed that the treatment with IGF-I but not with [QAYL]IGF-I (which has lower affinity for IGFBP and normal affinity for IGF-IR), released membrane-associated IGFBP-3 into the medium of cultured human breast cancer Hs578T cells. However the association of IGFBP-3 with GAGs does indeed occur (Arai *et al.*, 1994a) and thus it was investigated whether these polymers were responsible for IGFBP-3 association to the cell monolayer. In human skin fibroblasts and rat glioma cells, heparan sulfate and chondroitin sulfate account for all of the cell membrane associated GAGs. Pretreatment of the cell monolayer with heparitinase (which degrades heparan sulfate GAGs), or heparinase (which preferentially degrades highly sulfated GAGs like heparin and heparan sulfate) or ABC chondroitinase (which digests chondroitin sulfate) did not decrease ^{125}I IGFBP-3 binding to the cell monolayers (Yang *et al.*, 1996). Similar enzymatic treatments and also the addition of sodium chlorate to the culture medium failed to affect ^{125}I IGFBP-3 binding to Isikawa endometrial cancer cells (Karas *et al.*, 1997). Taken together these data indicate that the interaction of IGFBP-3 to the cell surface cells is unlikely to occur via GAGs. In contrast, Smith *et al.*(1994) showed that sodium chlorate treatment, which inhibits the enzyme responsible for sulphation of GAGs (Hoogewerf *et al.*, 1991), reduced the binding of exogenously added IGFBP-3 to rat Sertoli cells in a dose dependent manner.

Several reports show that not only does ^{125}I IGFBP-3 associate to the cell membrane, but, after incubation at 37°C, it becomes internalized (Yang *et al.*,1996; Karas *et al.*,1997) through an endocytotic process which can be inhibited by incubating the cells at low temperature (Smith *et al.*, 1994). Internalization and lysosomal degradation of cell associated IGFBPs could represent an additional aspect of the complex modulation of the IGF-IGFBP axis.

Inhibition of IGF action.

As reviewed in Clemmons (1997), IGFBP-3 added in excess to Balb/c3T3 cells in culture inhibited the insulin-like actions of IGF-I (Okajima *et al.*, 1993). In chick fibroblasts IGFBP-3 decreased IGF-I DNA synthesis (Blat *et al.*, 1989) and in rat granulosa cells the steroidogenesis and the cAMP rise stimulated by IGF-I was inhibited by exogenous IGFBP-3 (Bicsak *et al.*, 1990). IGFBP-3 inhibited collagen synthesis in rat and mouse osteoblasts and IGF-I stimulated glucose uptake in pig fat cells (but it was ineffective on

insulin-stimulated glucose uptake). It was proposed that the increased concentration of IGFBP-3 in the conditioned medium in mouse embryo fibroblasts could account for the density dependent inhibition of cell growth (Blat *et al.*, 1994). Nickerson *et al.*, (1997) reported that in MCF7 cells 72h incubation with IGFBP-3 caused a reduction of ^{3}[H]thymidine uptake and a 3.5 fold increase in the number of apoptotic cells. IGFBP-3 also antagonized the antiapoptotic effect of IGF-I, but not of R^{3}IGF-I, an IGF-I analog with full affinity for IGF-IR and reduced affinity for IGFBPs. This indicates that the apoptotic effect of IGFBP-3 in this cell line derives from its ability to prevent IGF interaction with IGF-IR. In a human glioblastoma cell line, A172, the induction of p53 gene expression caused apoptosis and the stimulation of IGFBP-3 gene promoter and protein expression. It has been shown that in this cell line the addition of recombinant IGFBP-3 causes apoptosis but it remains to be demonstrated if the p53-induced apoptosis is mediated through the increase of endogenous IGFBP-3(Shen and Glazer, 1998). Karas *et al.*(1997) showed that in Isikawa endometrial cancer cells membrane-associated IGFBP-3 inhibited short (IGF-IR and IRS-I phosphorylation) and longer term (induction of c-fos, increased AP1 binding to TRE oligonucleotides) signalling events.

IGF-potentiating action.

When either human recombinant glycosylated or non glycosylated IGFBP-3 was incubated with bovine fibroblasts, it associated to the cell monolayer and constituted an additional low affinity binding site for ^{125}I IGF-I. After 72h pre-incubation of the cells with IGFBP-3, the AIB and thymidine uptake stimulated by IGF-I, insulin and [QAYL]IGF-I was potentiated (Conover, 1992). Cell responsiveness to the growth factors correlated with their affinity for IGF-IR , independently from their affinity for IGFBP-3. It could be hypothesized that IGFBP-3 localizes IGF-I on the cell membrane and slowly releases it to the receptor, thus preventing the IGF-IR down regulation induced by an excess of IGF. However the potentiation of insulin and [QAYL]IGF could not be explained with this model.

IGF-independent effects.

Several lines of evidence have been collected in favor of an IGF-independent effect of IGFBP-3 in some cell culture models.

Oh *et al.* (1993c) reported that IGFBP-3 binds to the Hs578T cell surface and inhibits cell proliferation stimulated by IGF-I, but not by IGF analogs with reduced affinity for IGFBP-3. By cross-linking ^{125}I IGFBP-3 to the cell monolayer and subsequently immune precipitating the cell lysate with anti IGFBP-3 antibodies 3 cell surface proteins (20, 26 and 50 kDa) with IGFBP-3 binding properties were identified (Oh *et al.*, 1993b). The authors propose that the IGFBP-3 inhibitory effect on cell proliferation could be mediated through a specific IGFBP-3 receptor. In 1995 the same authors showed that substances that inhibited Hs578T cell growth, such as TGFβ_2 (tumor suppressor growth factor), antiestrogens and retinoic acid, induced an increase of IGFBP-3 expression. TGFβ_2-induced growth arrest was reversed by 60 % by transfecting the cells with antisense IGFBP-3. IGF-II and Leu^{27} IGF-II (which has full affinity for IGFBP-3 and reduced affinity for IGF-IR) were able to reverse TGFβ_2 stimulated growth suppression, but [QAYLL]IGF-II (which has full affinity for IGF-IR and reduced affinity for IGFBP-3) did not. These data indicate that the mechanism by which TGFβ_2 determines the reduction of the cell growth cannot be explained by the simple increase in IGFBP-3 and subsequent sequestration of IGFs from IGF-IR, and suggests that IGFBP-3 acts in an IGF-independent fashion (Oh *et al.*, 1995). Further support for this hypothesis came from a study where it was shown that IGF-IR-negative mouse fibroblasts, transfected with human IGFBP-3, displayed lower growth rate compared to the controls (Valentinis *et al.*, 1995). Moreover several reports have shown that IGFBP-3 fragments derived by limited plasmin proteolysis, despite their reduced affinity for IGF, inhibited IGF-stimulated mitogenesis and even the mitogenic effect of insulin (Oh, 1997). In the prostate cell PC-3, IGFBP-3 fragments derived by plasmin digestion displayed opposite biological effects. Intact IGFBP-3 was able to increase cell proliferation and $^{1\text{-}169}$IGFBP-3 fragment was almost three times more effective than the wild type molecule. This effect is probably due to a modulation of endogenous IGF-II-induced cell proliferation as it is markedly reversed by αIR-3, an anti IGF-IR monoclonal antibody. In contrast $^{1\text{-}95}$IGFBP-3 fragment, derived from further digestion of the 1-169 peptide, reduced cell proliferation to 50% of basal levels. This fragment (that does not bind IGF-II), was able to reverse IGF-II induced cell proliferation even when the accessibility of IGF-IR was blocked with the addition αIR_3 (Angelloz-Nicoud *et al.*, 1998). These data confirm previous work of the same research group which described the growth inhibitory effect of $^{1\text{-}95}$IGFBP-3 fragment in mouse fibroblasts with targeted disruption of IGF-IR gene (Mohseni-Zadeh and Binoux, 1997a).

The same authors also showed that intact IGFBP-3 is able to inhibit both IGF-I and des(1-3)IGF-I proliferative activity. The authors propose that in this case IGFBP-3 might bind directly to IGF-IR preventing its interaction with the IGF peptides (Mohseni Zadeh and Binoux, 1997b).

An alternative method of interaction between the cells and IGFBPs has recently been described for IGFBP-3 and 5 which were shown to translocate to the cell nucleus. With immunofluorescence IGFBP-3 was detected in the nucleus of proliferating human keratinocytes and laser scanning confocal microscopy confirmed that the binding protein was present in the nucleus and not in the perinuclear spaces (Wraight *et al.*, 1998). Nuclear uptake of fluorescently labelled IGFBP-3 and 5 was demonstrated in proliferating human breast cancer cells after 90'minutes whereas no nuclear uptake was observed after the addition of equivalent amount of labeled IGFBP-1 and 2 although all labeled proteins were shown to associate to the cell membrane (Schedlich *et al.*, 1998). Both IGFBP-3 (Radulescu, 1994) and 5 contain a NLS sequence (nuclear localization signal) inside the C-terminal 18 residue sequence (215-232 for IGFBP-3 and 210-218 for IGFBP-5) that is also involved in heparin/GAGs and ALS binding. The NLS motif is a bipartite nuclear targeting sequence found in many nuclear proteins and consists of 2 basic residues followed by an interval of 10-11 amino acids and then at least 3 basic residues within the next 5 positions. Substitution of the (^{228}KGRKR) IGFBP-3 sequence with the corresponding (MDGEA) IGFBP-1-derived sequence abolishes nuclear uptake (Schedlich *et al.*, 1998). In contrast to the IGFBP-3 internalization by endocytosis reported by Smith (1994), IGFBP-3 nuclear localization does not seem to be affected by lysosomotropic inhibiting agents nor by microtubule disrupting agents, suggesting the internalization and translocation occurs through an alternative pathway (Schedlich *et al.*, 1998). Nuclear translocation has been described also for insulin, platelet derived growth factor and fibroblast growth factor which stimulate cellular responses either by an indirect signal pathway or directly through nuclear targeting. A similar possibility could exist for IGFBP-3 and the choice between one or the other pathways might depend on the cell cycle phase (Schedlich *et al.*, 1998), as nuclear localization seems to occur only in cells in mitotic phase at the cytokinesis stage (Wraight *et al.*, 1998). In particular nuclear targeting could explain some of the cellular growth inhibitory effects of IGFBP-3 which do not appear to be IGF-mediated (Wraight *et al.*, 1998).

Finally although IGFBP-3 preferentially binds to the cell surface, but it can also associate to ECM, although less potently in comparison with IGFBP-5 (Jones *et al.*, 1993 a; Imai *et al.*, 1997).

1.2.2.b.3IGFBP-4

IGFBP-4 modulation on IGF action is mainly inhibitory, as reviewed in Jones and Clemmons (1995a) and Clemmons (1997).

Inhibition of IGF effects

In rat Leydig cells IGF-I enhances hCG-stimulated steroidogenesis and IGFBP-4 inhibits the effect of the growth factor (Lin *et al.*, 1993). IGFBP-4 inhibits human osteosarcoma cell growth and glycogen synthesis, when added in excess over IGF-I and IGF-II (Kiefer *et al.*, 1992) and an inhibitory effect on DNA synthesis has been reported also in colon carcinoma cells (Culouscou and Shoyab, 1991). In the B104 neuroblastoma rat cell line IGFBP-4 inhibits IGF-I stimulated, but not des(1-3)IGF-I-stimulated, ^{3}[H] thymidine uptake (Cheung *et al.*, 1991). IGFBP-4 antagonized IGF-I- stimulated AIB uptake or DNA synthesis in fibroblasts and in porcine smooth muscle cells (pSMC). The level of this inhibition was affected by specific IGFBP-4 proteases released in the conditioned medium by cultured pSMC and human fibroblasts (Cohick *et al.*, 1993; Conover *et al.*, 1993a). The principal cleavage site at which proteases produced by B104 neuroblasts cleave IGFBP-4, is located between K120 and H121, while a secondary cleavage site lies between M131-K132 (Chernausek *et al.*, 1995). The exposure of rat IGFBP-4 to human fibroblasts also causes proteolytic degradation and a cleavage site after M135 residue has been described. Consistently rat IGFBP-4 mutated in the vicinity of 132-135 sequence appeared more potent in inhibiting IGF-stimulated DNA synthesis in human fibroblasts (Conover *et al.*, 1995). The ability of IGFBP-4 or various IGFBP-4 derived or mutated proteins to antagonize IGF-II-stimulated proliferation in human osteosarcoma cells was tested. The inhibitory effect correlated with the affinity of each IGFBP-4 form for IGF-II. Thus IGFBP-4 and H74P IGFBP-4 mutant had equal affinity for IGF-II and the highest inhibitory action. A C-terminus-deleted IGFBP-4 molecule had reduced affinity for IGF-II and is also less potent in reducing the effect of the growth factor. In contrast the N-terminus $^{1-71}$ IGFBP-4,

which binds IGF-II very weakly, and the C-terminus of the molecule, which shows no affinity for IGF-II, were not effective in inhibiting IGF-II stimulated cell growth (Qin *et al.*, 1998).

1.2.2.b.4 IGFBP-5

IGFBP-5 shares structural and functional similarities with IGFBP-3 and, like IGFBP-3, can exert an inhibitory or enhancing effect on IGF action or may even have an IGF-independent function. The biological action of IGFBP-5 varies substantially depending on whether the binding protein is present in soluble form in the conditioned medium or is associated with the solid phase on the cell membrane or ECM. A summary of the data in this area will precede the description of the functions of IGFBP-5 in cell culture. As for IGFBP-3, it is believed that IGFBP-5 might either bind to GAGs or to the proteins normally present on the cell surface or on the ECM.

IGFBP-5 has been shown to interact with ECM proteins, such as type IV collagen and type III collagen, fibronectin and laminin, (listed in decreasing order of affinity), while it does not bind to vitronectin, type I, V and VII collagen (Jones *et al.*, 1993a). Moreover as IGFBP-5 binds to heparin (Andress, 1995), it was investigated if GAGs, which are structurally related to heparin, were also involved in the in IGFBP-5 interaction with the ECM/cell surface. It was found that the binding of IGFBP-5 to ECM prepared from human fibroblast or to mesangial cell monolayer was inhibited by high salt concentration, competed by heparin and decreased by enzymatic degradation of GAG residues with heparinase (Jones *et al.*, 1993a; Arai *et al.*, 1996 and Abrass et al., 1997). It was also shown that IGFBP-5 bound to plates coated with a purified heparan sulfate proteoglycan, tenascin, and the binding was reduced by heparinase treatment (Arai *et al.*, 1996). These results support the hypothesis that IGFBP-5 binding to the ECM occurs at least in part via GAGs, as well as through direct binding to ECM proteins. Andress (1995) tested if GAGs were also involved in the association IGFBP-5 with the cell membrane. Both IGFBP-5 and its C-terminal-truncated variant $^{1\text{-}169}$ IGFBP-5 bound to osteoblast-like cell membranes and became internalized. The binding was inhibited by high salt concentration and competed by heparin> heparan sulfate> dermatan sulfate in decreasing order for both proteins, although higher salt and heparin concentration were required to displace $^{1\text{-}169}$ IGFBP-5 compared to wild type protein. However, these results derived from a direct competition between the above molecules and the cell membrane GAGs,

were questioned by the inability of the heparinase or sodium chlorate treatment of the cell monolayers to affect IGFBP-5 binding to the cell membrane. By cross-linking IGFBP-5 or $^{1\text{-}169}$ IGFBP-5 to osteoblast monolayers, it was found that the tracer proteins formed a complex with a 420kDa Triton-extractable membrane protein and this protein was then purified from a cellular extract on an IGFBP-5 affinity chromatography column. The affinity labeling was greater for IGFBP-5 than for its truncated variant and the formation of the complex was not affected by heparinase or chondroitinase pretreatment of the cell monolayer. In contrast IGFBP-5 and $^{1\text{-}169}$IGFBP-5 failed to cross-link to any protein of an ECM preparation. In conclusion, while IGFBP-5 association to the ECM might involve GAG interactions, the binding to osteoblast cell surfaces was mediated by a 420kDa protein, which would serve as an IGFBP-5 specific receptor. Its functions as a receptor were further investigated in a recent study by the same author (Andress, 1998). The treatment with IGFBP-5 or with the 201-218 peptide derived from IGFBP-5, caused an increased serine phosphorylation of the 420kDa protein which in addition displayed some kinase activity itself as it was able to induce serine phosphorylation of casein *in vitro*.

When a nuclear localisation sequence (NLS), similar to the one described for IGFBP-3, was identified also in IGFBP-5 (Schedlich *et al.*, 1998), an alternative route for IGFBP interaction with cells emerged. In IGFBP-5 the nuclear translocation sequence is located in the 201-218 region which is also involved in the binding to heparin, ALS and PAI. More details on nuclear translocation have been described in the section detailing the IGF- independent effects of IGFBP-3.

Finally it is important to remember that the microenvironment can modulate the IGF-IGFBP axis in many different ways. For example,

-While IGFBP-3 preferentially binds to the cell surface, IGFBP-5 preferentially binds to the ECM (Jones *et al.*, 1993a, Imai *et al.*, 1997)

-When IGFBP-5 is bound to heparin or to the ECM its affinity for IGFs is decreased 8-15 fold while IGFBP-3 affinity for IGFs shows a 12-fold decrease when the binding protein is associated to the cell surface, but not when it is bound to ECM (McCusker *et al.*, 1990; Arai *et al.*, 1996; Arai et al., 1994a).

-In many cell culture conditions extensive proteolytic activities degrade IGFBP-5 and the resulting fragments show a reduced binding for IGFs (Camacho-Hubner *et al.*, 1992).

-The proteolytic activity is generally inhibited when IGFBP-5 is bound to the ECM or to IGF-I (Jones *et al.*, 1993a; Camacho-Hubner *et al.*, 1992 and Sunic *et al* ., 1998).

IGF inhibiting effects.

IGFBP-5 inhibited IGF-I and II-stimulated DNA and glycogen synthesis in human osteosarcoma cells (Kiefer *et al.*, 1992). Moreover IGFBP-5 decreased the IGF-I stimulated steroidogenesis in Leydig cells (Ling *et al.*, 1993).

In some of the models used to investigate the biology of this binding protein it was noted a correlation existed between the occurrence of apoptosis and the increased expression of IGFBP-5. Guennette and Tenniswood (1994) reported that IGFBP-5 expression increases in involuting prostate and suggest that the binding protein associated with the ECM would prevent IGF interaction with the IGF-IR and therefore the subsequent delivery of IGF survival signals. A contemporary increase in IGFBP-5 expression and in apoptotic cell number was also noted in finasteride or castration-induced prostate involution, although differential staining for DNA breakage or for IGFBP-5 expression did not overlap within the same tissue sections (Thomas *et al.*, 1998). A correlation between IGFBP-5 expression and apoptosis was also observed in atretic follicles (Erickson et al., 1992) and in rat involuting mammary gland (Tonner *et al.*, 1997).

IGF potentiating effects

Potentiating effects of IGFBP-5 have been reported by several authors in fibroblasts, bone cells and smooth muscle cells (SMC).

ECM-associated IGFBP-5 enhanced human fibroblast growth in response to IGF-I treatment, while the binding protein in the CM was degraded to a 21kDa fragment lacking of potentiating effects (Jones *et al.*, 1993a). In human glioblastoma cells (T98G) endogenous IGFBP-5 was associated with the cell membrane and showed 5 fold higher affinity for IGF-II, than for IGF-I. Zn^{++} induced a reduction in the affinity of membrane-associated IGFBP-5 for IGFs but at the same time it stimulated retention of IGFBP-5 on the cell surface. In this system Zn^{++} was able to potentiate IGF action in the absence of exogenously added IGFBP-5. Therefore the Zn^{++}potentiating effect might derive from a facilitated release of IGF from the cell surface-associated IGFBP-5 to the IGF-IR (Sackett and McCuscker, 1998). In bone

cells, as well as in smooth muscle cells, IGFBP-5 has been reported to have alternatively an effect promoting cell proliferation or cell differentiation. In human osteoblast-like cells both IGFBP-5 and its carboxy-truncated 23kDa fragment enhanced IGF-I and IGF-II stimulated mitogenesis. IGFBP-5 stimulated cell proliferation in the absence of exogenous or endogenous IGFs (Andress and Birnbaum, 1992). During the differentiation process in MC3T3-E1 mouse osteoblasts, IGFBP-5 secretion increases in the initial cell proliferation period and it reaches its peak at transition with the start of differentiation, then it declines. In contrast, it has been noticed that in rat osteoblasts, growth factors which stimulated cell proliferation, such as FGF, TGFβ and PDGF, decreased the expression of IGFBP-5, IGF-I and IGF-II (Canalis and Gabbitas, 1995; Canalis *et al.*, 1993), while factors that stimulated cell differentiation, (namely IGFs and retinoic acid), increased IGFBP-5 expression (Conover and Kiefer, 1993; Dong and Canalis, 1995; Gabbitas and Canalis, 1998). Cortisol which is an inhibitor of multiple parameters of bone formation, including cell proliferation, reduced IGFBP-5 expression and its deposition onto the ECM (Gabbitas *et al.*, 1996).

As with bone cells, also in smooth muscle cells (SMC) contrasting results have been published suggesting that IGFBP-5 potentiated either the proliferating or the differentiating effect of IGF peptides. In pSMC transfected with IGFBP-5 cDNA the expressed protein accumulated in the ECM more abundantly than in the control cells and it potentiated the IGF-I stimulation of DNA synthesis. Conversely, the transfection of pSMC cells with IGFBP-5 mutants which have reduced ECM binding affinity, resulted in an inhibition of IGF-I stimulated ^{3}[H]Thymidine uptake (Parker *et al.*, 1998). The presence in conditioned medium of a low level of intact IGFBP-5 with high affinity for IGF, together with the presence of abundant intact ECM-associated IGFBP-5 with reduced affinity for IGFs appears to be the condition required for the binding protein to exert an IGF potentiating effect. The authors propose that IGFBP-5 should be included in the group of the crinopectins, molecules which interact with proteoglycans and facilitate the interactions between the growth factors and their receptors (Parker *et al.*, 1998).

Similar results in terms of potentiation of IGF stimulated ^{3}[H]thymidine uptake were obtained in SMC when monolayers were incubated for 48h with IGFBP-5, but not after a 24h incubation period. It was suggested that, in order to achieve an IGF enhancing effect IGFBP–5 required to be proteolyzed into a 21kDa fragment, which retained the ability to associate to the ECM but had greatly reduced affinity for

IGFs. This theory is further strengthened by the observation that a proteolysis resistant IGFBP-5 mutant in the same culture conditions revealed an IGF inhibitory effect (Duan and Clemmons, 1998). In this latter study it was shown that IGFBP-5 and IGF expression is high in proliferating cells and declines when cells reach confluence. In contrast other authors report that in L6A1 muscle cells (Ewton *et al.*, 1998) and in C2 mouse myoblasts (Rousse *et al.*, 1998) IGFBP-5 expression increases during cell differentiation.

Rousse *et al.*(1998) showed that in the same C2 cell line, stably transfected with antisense IGF-II, IGFBP-5 expression was indeed increased during myogenic differentiation, but this phenomenon was independent from the differentiation process and directly regulated by IGF-IR activation. In contrast, evidence of a direct involvement of IGFBP-5 in myogenic differentiation was provided by studies showing that C2 myoblasts overexpressing IGFBP-5 failed to differentiate, while the transfection of the cells with antisense IGFBP-5 causes earlier differentiation.(James *et al.*, 1996).

In L6A1 cells IGFBP-5 expression was higher in differentiating cells and the binding protein showed an inhibitory effect on IGF stimulated proliferation and a potentiating effect on cell differentiation stimulated by IGF-I but not IGF-II (Ewton *et al.*, 1998).

Finally it should be pointed out that in many experimental models the IGF-IGFBP axis cannot really be considered as an isolated system. For example recent work showed that the SMC phenotype can be substantially influenced by the type of substrate the cells are cultured on. On laminin and collagen the cells display a dedifferentiated phenotype, while on fibronectin the cells show a synthetic phenotype and respond to growth factor stimulation with an increased cell proliferation or migration. Cells cultured on fibronectin produced 4 times more IGFBP-5, than cells cultured on laminin/collagen. Moreover the addition of exogenous IGFBP-5 did not affect cells seeded on fibronectin whereas it markedly increased IGF-I-stimulated DNA synthesis in cells plated on laminin/ collagen. Thus it appears that ECM proteins, by interacting with cell membrane integrins, can influence the IGF-IGFBP-5 axis. In particular this study showed that ligand occupancy of $\alpha_v\beta_3$ by vitronectin or thrombospondin induces IGF signalling events which lead to the stimulation of cell proliferation or cell migration (Zheng *et al.*, 1998b).

IGF-independent actions.

IGFBP-5 and an IGFBP-5 derived peptide containing the 201-218 sequence were shown to stimulate cell proliferation in mesanglial cells (MC), but in the presence of IGF-I, IGFBP-5 reduced IGF-

induced cell migration. MC cells express $\alpha_v\beta_3$, and $\alpha_5\beta_1$ integrins. The addition of kistrin, a disintegrin which binds to $\alpha_v\beta_3$ integrin, abolished IGF-I induced migration, but not migration stimulated by 201-218 peptide. Therefore it seems that IGF and IGFBP-5 stimulate cell migration through 2 different mechanisms (Abrass *et al.*, 1997).

1 2.2.b.IGFBP-6

Due to its low affinity for IGF-I, IGFBP-6 inhibitory effect is mainly restricted to IGF-II action.

Inhibition of IGF effects.

In the ovary FSH and LH stimulate IGFBP-6 secretion which in turn inhibits IGF-I stimulated steroidogenesis, although less potently then IGFBP-2 or 4 (Jones and Clemmons, 1995a). IGFBP-6 inhibits IGF-II, but not IGF-I, induced differentiation in myoblasts (Bach *et al.*, 1994). In MC3T3-E1 osteoblasts IGFBP-6 reduced the basal level of ^{3}[H] thymidine uptake, abolished the IGF-II-induced increase in thymidine incorporation, but inhibited only 40% of the IGF-I induced effect. The higher affinity of IGFBP-6 for IGF-II compared to IGF-I probably accounts for the difference in inhibitory strength. (Srinivasan *et al.*, 1996). As described for other IGFBPs, IGFBP-6 expression can be subjected to TGFβ regulation which has been shown to decrease IGFBP-6 mRNA in osteoblasts (Gabbitas and Canalis, 1997).

IGFBP-6 secretion in keratinocytes, myoblasts and neuroblastoma cells increases when cells undergo growth arrest. Retinoic acid in neuroblastoma cells induces differentiation, reduces IGFBP2 and 4 expression, but increases IGFBP-6 expression, whose function might be to sequester endogenous IGF-II from the interacting with IGF IR in these cells (Chambery *et al.*, 1998). From *in vitro* and *in vivo* studies some indications suggest that through the same mechanism IGFBP-6, which is expressed in a range of cell tumours (breast carcinoma, prostate carcinoma, endometrial carcinoma, neuroblastoma) might play a growth inhibition role (Sheikh *et al.*, 1993; Bach and Rechler, 1996; Drivdahl *et al.*, 1995; Gong *et al.*, 1992; Babajko and Binoux, 1996).

1.2.2.b.6.IGFBP-2

An IGF-inhibitory effect is generally attributed to IGFBP-2, however a correlation between IGFBP-2 plasma concentration and cell proliferation in patients affected by cancer has been reported *in vivo* and a few enhancing effects have also been described *in vitro*. As for other IGFBPs the association of IGFBP-2 to the cell membrane or to the ECM might be biologically significant and a review of the information available in the literature will precede the description of IGFBP-2 effects in cell culture.

As described above, IGFBP-2 has an RGD sequence in the C-terminus of the molecule, but unlike IGFBP-1 which was shown to bind to $\alpha_5\beta_1$ integrin, no binding to integrin has been demonstrated for IGFBP-2. Although the possibility that this binding occurs cannot be excluded, alternative mechanisms of interaction between IGFBP-2 and the cell membrane have been investigated. IGFBP-2 is secreted in many tissues and cell lines, but its association to the cell membrane has been poorly reported. Within the rat brain only in the olfactory bulbs is IGFBP-2 largely bound to the cell membrane, although it is produced throughout the whole brain (Russo *et al.*, 1997). Similarly in non small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) derived cell lines which both secrete IGFBP-2, the association of the binding protein with membranes is more abundant in SCLC cell lines (Reeve *et al.*, 1993). The association of IGFBP-2 with the cell membrane was demonstrated with several different assays. Many studies were carried out with [^{125}I] IGFs affinity labeling experiments where the radiolabelled growth factors were cross-linked either to cell membrane preparations or directly to cell monolayers which produced IGFBP-2. The electrophoretic resolution of the cross-linked material revealed two bands corresponding to the complexes between tracer and the IGF-I or -II receptors, and their appearance was competed by cold IGFs or des(1-3)IGF-I. In addition a band of approximately 40 kDa was apparent, which was competed by cold IGF-I or –II, but not by insulin or des(1-3)IGF-I which do not bind IGFBPs (Reeve *et al.*, 1993; Russo *et al.*, 1995; Bradshaw *et al.*, 1999). Based on the size of the protein, the IGF competition pattern and the IGFBP expression profile of the cells used in the experiments, this membrane-associated IGFBP was inferred to be IGFBP-2. More direct evidence of the presence of an IGFBP species on the cell membrane came from a direct ^{125}I IGF-I ligand blot of cell membrane preparations (Russo *et al.*, 1995). The actual identification of the membrane associated IGF-binding protein species as IGFBP-2 was achieved with an anti IGFBP-2 Western blot of a crude membrane

preparation (Reeve *et al.*, 1993) or by immune precipitation of solubilized membranes with anti IGFBP-2 antibodies (Reeve *et al.*1993; Bradshaw *et al.*, 1999). The next step was to try to identify the nature of the IGFBP-2 binding molecule on the cell membrane. Arai *et al.* (1996) showed that IGFBP-2 could bind to heparin Sepharose only when it was precomplexed with IGF-I or II and this binding could be competed by cold heparin or heparan sulfate, but only weakly by chondroitin sulphate A, dermatan sulphate or chondroitin sulphate C. It was also shown that IGFBP-2 bound to human fibroblast ECM and IGF-II was able to increase the binding by 18-fold. These results conflict with a later study carried out by Russo *et al.* (1997) who showed that IGFBP-2 could bind to heparin, heparan sulphate, chondroitin sulphate, keratan sulphate and aggrecan (a proteoglycan containing chondroitin and keratan sulphate residues) in the absence of IGFs. The discrepancies between the two studies could derive from the different type of assays or from the different IGFBP-2 species used in these experiments. The treatment of aggrecan-coated plates with chondroitinase or keratanase decreased IGFBP-2 binding, supporting the hypothesis that GAG residues of aggrecan played a part in this interaction. Consistently, NaCl and heparin were able to inhibit cross-linking of ^{125}I IGF-I with soluble or membrane-associated IGFBP-2, while RGD peptides were ineffective, suggesting that the RGD sequence of IGFBP-2 is not involved in the interaction with the cell membranes and that probably the binding is not mediated by integrins (Russo *et al.*, 1997 Bradshaw *et al.*, 1999). Finally it was demonstrated that in solubilized membrane preparations (from rat olfactory bulbs) an anti IGFBP-2 antibody was able to coimmuno-precipitate IGFBP-2 complexed with an unidentified molecule. The complex was separated in electrophoresis and the molecule bound to IGFBP-2 appeared to be a proteoglycan which migrated with an apparent Mr of about 200kDa which was recognized in Western blot by anti chondroitin sulphate antibodies (Russo *et al.*, 1997).

In conclusion IGFBP-2 was shown to associate to the ECM and the cell membrane and some indications support the hypothesis that this binding is mediated by GAGs.

Inhibitory effects of IGF action.

In most of the cell culture systems studied, IGFBP-2 accumulates in the conditioned medium where it binds to IGFs with high affinity and inhibits IGF action by preventing the interaction of the growth factors with the IGF-I receptor.

In rat calvaria cells IGFBP-2 reduced basal, and IGF-I-stimulated, ^{3}[H]thymidine uptake and collagen synthesis (Feyen *et al.*, 1991). In porcine muscle satellite cells, multiple passaging culture caused reduced cell division and myotube formation accompanied by an increased secretion of IGFBP-2, which may reduce endogenous IGF-I activity (Fligger *et al.*, 1998). The addition of equimolar concentrations of soluble IGFBP-2 and IGF-II to the NSCLC cell line caused a reduction in DNA synthesis compared with IGF-II treatment alone. However no differences between the two treatments were revealed in the SCLC cell line. Consistently, soluble IGFBP-2 was less efficient in antagonizing IGF-II binding to SCLC cells, in which IGF-II tracer bound to IGF-IIR and to cell surface associated IGFBP-2, in comparison to NSCLC cells in which radiolabelled IGF-II mainly associated to IGF-IR (Reeve *et al.*, 1993).

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

In IEC-6 rat intestinal cells, ^{3}[H]thymidine uptake and ^{14}C leucine incorporation were stimulated more potently by des(1-3)IGF-I and QAYL-IGF-I (IGF analogs with wild type affinity for the IGF-IR and reduced affinity for IGFBPs) than the wild type IGFs. This suggested that the endogenously secreted IGFBP-2 could account for the observed difference in IGF activity (Park *et al.*, 1992). Treatment of the same cell line with Na butyrate caused apoptosis and stimulated the expression of some genes, including IGFBP-2, believed to be involved in the apoptotic process (Guo *et al.*, 1998). The inhibition of IGFBP-2 expression by the transfection of IEC-6 cells with an anti-sense IGFBP-2 construct, stimulated and increased cell proliferation in comparison with the control cells (Corkins *et al.*, 1995).

Analogous experiments were undertaken in kidney fibroblasts stably transfected with an IGFBP-2 expression vector. The cell growth rate of cells transfected with IGFBP-2 was reduced compared to the control cells and the inhibition was reversed by IGF-I and II and even more potently by LongR3 IGF-I which has reduced affinity for IGFBPs. The anti-proliferating effect of IGFP-2 was confirmed when the

medium conditioned by IGFBP-2-transfected kidney fibroblasts was applied to choriocarcinoma cells, causing a reduction of cell proliferation (Höflich *et al.*, 1998)

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

Some studies on the effect of retinoic acid on cultured cells also support the inhibitory role of IGFBP-2. In a bovine mammary gland cell line, MAC-T, cultured in 1%FBS, retinoic acid inhibited cell proliferation and the expression of IGFBP-3, while it stimulated an increase in IGFBP-2 levels in the conditioned media and on the cell surface. Retinoic acid-induced growth inhibition was reversed by IGFs and even more efficiently by des(1-3)IGF-I indicating that at least in part the retinoic acid effect was mediated by the increased secretion of IGFBP-2 (Woodward *et al.*, 1996). It should be mentioned that in

other cell lines, eg. neuroblastoma cells, retinoic acid depressed IGFBP-2 expression (Chambery *et al.*, 1998).

Potentiating effects on IGF action.

Little evidence has been reported on a possible IGFBP-2 enhancement on the IGF effect. As indicated above, a correlation between high IGFBP-2 expression level and active tumour processes has been reported by several authors (Elmlinger *et al.*, 1998, Mishra *et al.*, 1998; Cohen et al., 1993; Fuller *et al.*,1999; Ho and Baxter, 1997; Kanety *et al.*, 1993, Kanety *et al.*, 1996; Karasik *et al.*, 1994). Recently it was shown that in both normal and malignant colorectal tissue, IGFBP-2 is proteolysed into a 22 and 30kDa fragments, but mRNA and protein expression of IGFBP-2 is significantly increased in malignant tumour areas (Mishra *et al.*, 1998). Elevated IGFBP-2 concentration was noted in the serum of patients suffering from T cell leukemia (Blum *et al.*, 1993; Mohnike *et al.*, 1996). In a leukemic T cell line Molt4, the addition of IGF-II caused an increase in IGFBP-2 mRNA and protein secretion. Conversely, blocking endogenous IGF-II with an anti IGF-II antibody caused partial inhibition of the growth rate and the complete cessation of IGFBP-2 secretion (Elmlinger *et al.*, 1998). In neuroblastoma cells endogenous IGF-II mediates cell proliferation under the regulation of IGFBPs. It has been suggested that a partial proteolysis of IGFBP-2 could facilitate the release of IGF-II from the binding protein enabling the growth factor to stimulate cell growth (Menouny *et al.*, 1997).

In non tumour models IGFBP-2 was able to increase 2.2 fold the IGF-I stimulated ^{3}[H] thymidine uptake in pSMC (Bourner *et al.*, 1992) while in microvascular epithelial cells, a partially purified preparation of the binding protein increased IGF-I stimulated AIB uptake (Bar *et al.*, 1989).

IGF independent effects.

To our knowledge the only report on the possible IGF-independent effect of IGFBP-2 was published on UMR10601 rat osteoblasts-like cells.

IGF-I and -II treatment of these cells did not stimulate cell proliferation and decreased the GH binding with endogenous GHR. In contrast, both IGFBP-2 and -3 at 1μg/ml stimulated GH binding to its receptor and displayed a marked mitogenic activity on these cells (Slootweg *et al.*, 1995).

In conclusion it would appear that IGFBP-2 displays mainly an anti proliferating effect whether present as a soluble form in the conditioned medium or associated to cell membranes.

1.3. Aims and objectives

At the beginning of our studies, an important scientific debate in IGFBP research was focused on the attempt to define the relative contribution of the N-terminal or C-terminal IGFBP domain to IGF binding. Observing the aligned N-terminus sequence of different species and different classes of IGFBPs, we noticed a group of very conserved proline residues (corresponding to residues at position 20, 21 and 22 in rat IGFBP-2. See Fig. *3.1.3.2*). We hypothesized that these amino acids might have escaped evolutionary re-modelling because they were involved in a specific biological function. Therefore we started our project testing what effect on IGF-binding was exerted by the introduction of two point mutations in that region of the rat IGFBP-2 sequence (section 3.1 of the results chapter). IGFBP-2 was initially chosen among other IGFBPs because we considered that its lack of post-translational modifications and the availability of commercial reagents (antibodies, purified IGFBP-2) could facilitate protein expression and identification. Each of two conserved N-terminal proline residues at position 20 and 22 were substituted with an alanine. The two mutants and the wild type proteins were produced with the baculovirus expression system and called P20A, P22A and wt IGFBP-2, respectively. Finally we compared IGF affinity of the wild type IGFBP-2 and its two mutants using the charcoal binding assay.

Although the IGF-binding site of IGFBPs still remains incompletely characterised, while we were working at our project, research had been making a remarkable progress and in the scientific literature a large amount of new intriguing findings in the IGFBP field were published. It became clear that in a biological context IGFBP activity did not only depend on affinity between IGF and IGFBPs, but was influenced by multiple factors (association with cell surface/ECM and proteolytic activities). Therefore we refocused our studies investigating other features of IGFBP-2 that could be relevant in understanding its biological function.

The best known effect of IGFBPs is an inhibition of IGF action. However, IGF-enhancing/independent effects have also been described for some IGFBPs and their association with the cell membrane/ECM appeared to be required for the occurrence of these effects. As far as IGFBP-2 is

concerned, its inhibitory action has been widely described, whereas only a very few studies reported its possible IGF-potentiating effect. However, a correlation between tumour cell proliferation and high IGFBP-2 expression level has been observed in an increasing number of studies. In addition, Guennette and Tenniswood (1994) theorized a model to explain the specific role played by different IGFBPs during the involution of the prostate gland. In this model it was postulated that IGFBP-5, which is expressed when the prostate undergoes involution, could exert a pro-apoptotic role by sequestering the IGFs in ECM and preventing their binding to IGF-IR. Conversely, IGFBP-2, which is constantly expressed before and during the involution process, could associate to the cell membrane and facilitate IGF interaction with its receptor allowing the delivery of a cell survival signal. Studies such as these led us to investigate alternative functions of IGFBP-2 in a suitable cell culture system.

As for other IGFBPs association with the cell membrane seemed to play a key role in IGF-potentiating effects, we first worked to identify a cell line in which IGFBP-2 binding to the plasma membrane could be demonstrated (section *3.3.1* of the results chapter). In this chapter the possibility that IGFBP-2 could bind to plasma membrane integrin receptors through its RGD sequence was also considered. RGD stands for arginine, glycine, aspartic acid and it is a recognition sequence for integrin receptors. To test if IGFBP-2 RGD sequence was involved in plasma membrane binding we also expressed and purified a PGD IGFBP-2 mutant, whose RGD sequence was altered by the substitution of the arginine residue with a proline and it was used as negative control in integrin-binding experiments.

After screening several cell lines and using various techniques, we succeeded in detecting the association of endogenous IGFBP-2 to plasma membranes of a rat liver cell line, Clone 9 cells.

We then demonstrated that this cell line is responsive to IGF stimulation and that it secretes IGF-I endogenously (section *3.3.2* of the results chapter).

Therefore Clone 9 cells met all the requirements as a suitable model for studying IGFBP-2 modulation of IGF action *in vitro*. In the last part of our project (section *3.3.3*) we proceeded to the transfection of Clone 9 cells with an antisense IGFBP-2 construct. Our aim was to compare wild type and antisense IGFBP-2 transfected cells with respect to their responsiveness to endogenous or exogenous IGF stimulation.

2. MATERIAL AND METHODS

General laboratory chemicals and reagents

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

2.1 Construction of P20A and P22A IGFBP-2 mutants, expression of wt IGFBP-2 and mutated proteins and assessment of their affinity for IGFs

2.1.a Composition of buffers, solutions, cell culture media used in molecular biology methods and a description of general techniques

TBE x10: 1M Tris-HCl, 0.9M Boric acid, 0.01M EDTA, pH 8.4

TAEx10: 0.4M Tris, 0.2M Na acetate, 0.01M EDTA, pH7.2 with glacial acetic acid.

5xOrange G loading buffer: 20ml 500 mM EDTA, 20g Ficoll, water to 100ml, Orange G (approximately 0.1g)

TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA). Autoclaved

Restriction digestion. A typical restriction digestion would be performed in excess of enzyme: 20μl reaction volume containing 1μgDNA, 2μl x10buffer (containing 0.5mg/ml BSA), 10units enzyme.

Endonuclease enzymes and appropriate 10x buffers were supplied by Boehringer or Promega. Digestions were incubated at recommended temperature for 1-2h.

Agarose gels

1% w/v *high gelling temperature agarose* (Promega) contained 1xTBE buffer and 0.5μg/ml of ethidium bromide (Biorad, Hertfordshire,UK). Before loading, DNA samples, typically in 10-15μl volume, were mixed with 2-3μl orange G loading buffer. DNA size markers used were λ DNA/Hind III fragments and φX174 RF/HaeIII fragments (Life Technologies). Electrophoresis was performed in 0.5xTBE for 90-120min at 80V using Electro4 or mini gel Hybaid (Ashford, Middlesex, UK) apparatus.

1% w/v *low melting point agarose* (Sigma) was dissolved in 1xTAE buffer and 0.5μg/ml ethidium bromide was added. Gels were electrophoresed in 1xTAE buffer as described above. Low melting point agarose gels were used for DNA inserts, that were to be excised from the gel and extracted with Geneclean^{R}II kit (Anachem, Luton, UK) following the instructions described by the manufacture. Electrophoresed DNA was visualised on a Herolab transilluminator (Mididoc, gel documentation analysis system and EASI store software Herolab Molekulare Trenntechnik,)

QUIA quick gel extraction filters (Qiagen, Crawly, West Sussex, UK): this kit was used either to purify linearized and dephosphorylated DNA plasmids in solution or to extract DNA inserts from high gelling point agarose gels. Purification was performed as recommended by the manufacturer.

Ligation a typical ligation reaction would be performed in 15μl volume containing: 1.5μl x10 ligation buffer (500mM Tris-HCl (pH7.5), 100mM $MgCl_2$, 100mM DTT, 10mM ATP, 250μg/ml BSA); 100-200ng of linearized, dephosphorylated plasmid DNA, 100-200ng of DNA insert with compatible restriction enzyme ends and 15U T_4DNA ligase (0.5μl of high concentration T_4DNA ligase from New England Biolab, Hitchin, Hertfordshire, UK). Reactions were incubated at 16°C overnight.

Competent cells JM109 (Promega) was the strain of *Escherichia coli* (*E. coli*) used for general transformations to clone DNA inserted in pGEM and pALTER plasmids. This strain is *endA*1, *recA*1, *gyrA*96, *thi*, *hsdR*17 (r_k^-,m_k^+), *relA*1,*supE*44,λ$^-$,Δ*(lac-proAB)*,[F',*traD*36, *proA*$^+$*B*$^+$, *lacI*^{q}ZΔM15].

LB (Luria Bertaini) medium: 10g Peptone, 5g yeast extract, 5g NaCl, H_2O to 1litre, pH7.5 and then autoclaved. Powders for bacteriological purposes were from Oxoid (Hampshire, UK). If antibiotic supplementation was required, medium was cooled to 55°C before drug addition. For the most commonly

used media, ampicillin or tetracycline was added to LB medium to a final concentration of 125μg/ml and 12.5 μg/ml respectively.

LB-agar plates 15g of agar were added to 1litre LB medium, pH adjusted to 7.0 and autoclaved. Drugs, if required, were added, at the concentration indicated above, when LB-agar solution reached 55°C. Agar was immediately poured onto 10 cm Petri dishes and, after setting, dried in a flow cabinet. Plates were stored at 4°C for up to one month (wrapped with foil for those plates which contained tetracycline).

Antibiotic stock solutions: ampicillin (125mg/ml), gentamycin (7mg/ml) and kanamycin (10mg/ml) were dissolved in water and filter-sterilized. Tetracycline was dissolved in 80% ethanol at 12.5mg/ml. All solutions were stored at –20°C in the dark.

SOC medium: 2g Tryptone, 0.5g yeast extract, 1ml of 2M NaCl and 0.25ml 1M KCl were added to 97ml H_2O, stirred until dissolved, autoclaved then cooled to RT. Then, 1ml of 2M Mg^{++} stock (prepared as followed: 1M $MgCl_2 \bullet 6H_2O$, 1M $MgSO_4 \bullet H_2O$, filtered sterilized) and 1ml of 2M glucose (filter sterilized) were added. The volume was adjusted to 100ml (pH7.0) and the solution was filter sterilized through a 0.2μm filter unit.

Transformation For a typical transformation, aliquots of JM109 competent cells would be thawed on ice for approximately 10min. 100μl of cell suspension would be placed in prechilled polypropylene tubes and after the addition of 50ng of plasmid DNA, tubes would be mixed and incubated on ice for 30min. Tubes were incubated at 42°C for 50seconds and immediately chilled on ice for 2min. After the addition of 900μl LB medium to each tube, cells would be left to recover at 37°C for 1h with shaking. 100μl of cell suspension would be plated onto LB-agar plates in the presence or absence of appropriate drug selection and plates incubated at 37°C for 18-24h.

Minipreps Typically minipreps were prepared as follows, although some minor difference were adopted when following protocols recommended by different manufactures.1.5ml of overnight bacterial cultures were centrifuged for 5min at 13000rpm on a bench top micro centrifuge (MSE microcentaur centrifuge). Cell pellets were resuspended in 100μl of prechilled *solution I* (25mM Tris- pH8.0, 10mM EDTA, 50mM glucose 2mg/ml lysozyme), mixed by vortexing and incubated on ice for 30min. 200μl of *solution II* (1% SDS, 0.2M NaOH), stored at RT, was added to the tubes, which were then gently mixed

by inversion and incubated on ice for a further 5min. Finally 150µl of prechilled *solution III* (3MNa acetate pH 4.8) was added. Tubes were mixed vigorously and a copious precipitate formed. After 45min incubation on ice and 5min centrifugation at 13000rpm, 400µl of the resulting supernatant was transferred to fresh tubes, avoiding the white precipitate. The addition of 2.5 volume of 100% ethanol and the incubation of the sample at –70°C for 30min-1h, would then lead to DNA precipitation. Tubes were then centrifuged for 10min at 13000rpm, ethanol was immediately aspirated, pellets washed with 70% ethanol and dried in a vacuum. Pellets were resuspended in 30µl RNAse (20µg/ml in H_2O).

If DNA was to be further purified phenol/chloroform extraction was undertaken before ethanol extraction. In this case the 400µl supernatants (resulting from the centrifugation which follows the addition of solution III) was supplemented with 1ml of phenol:choloroform:isoamyl alcohol (25:24:1), mixed for 1min and centrifuged at 12000x g for 5min. After transferring the upper aqueous phase to fresh tubes, 1ml of chloroform:isoamyl alcohol (24:1) was added. Mixing of the samples, centrifugation and transfer of the upper aqueous phase was repeated as before. DNA was precipitated and pelletted as described above.

Ultraclean mini plasmid prep kit (MoBio Laboratories, Solana Beach, Ca) was used in later experiments. The procedure used was that recommended by manufacture.

Maxipreps (QUIAGEN tips 500) were prepared following the method described by the manufacture

2.1.1. Site directed mutagenesis to create the cDNA encoding for P20A and P22A IGFBP-2 mutants

In order to introduce the mutations necessary for creating P20A and P22A mutants at specific sites in the IGFBP-2 cDNA, we employed the Promega "Altered sites^R II" *in vitro* mutagenesis system. This technique exploits the hybridization between the wild type gene and a single stranded oligonucleotide which has a sequence complementary to the wt gene, but which carries alteration of a few base pairs at the site of mutagenesis. Overview of the method: the IGFBP-2 gene was cloned in pALTERex1 plasmid which contains an inactivated ampicillin resistance gene and an active tetracycline resistance gene. In each mutagenesis reaction, three different oligonucleotides were used: i) one oligo to introduce the desired mutation (P20A or P22A), ii) one oligo to repair the ampicillin resistance gene, and iii) one oligo to knock

out the tetracycline resistance gene. T_4DNA polymerase was used to fill the gaps between the oligonucleotides, by using plasmid single strand DNA as template, and DNA ligase was used to seal together the newly synthesised DNA fragments. As a result, wt-mutant hybrid DNA duplex was obtained. In order to avoid the host-directed mismatch repair system which would repair the unmethylated newly synthesised DNA strand, the hybrid duplex was amplified in a *E. coli mutS* strain (ES 1301). Due to the semi-conservative mode of DNA replication, wt and mutated plasmids would theoretically be amplified at the same ratio. The introduction of ampicillin selection allowed an increase in the yield of mutated DNA over the wt.

PGEM 7Zf(+/-) rat IGFBP-2 was a kind gift of Dr Sean Guenette, John Wayne Cancer Institute Los Angeles.

The recombinant plasmid was amplified in JM109 cells under ampicillin selection. A description of methods followed for plasmid transformation into JM109cells, mini and maxi preps is as reported above.

IGFBP-2 cloning into pALTER ex1. The rat IGFBP-2 cDNA was excised from the pGEM vector as a BamHI-XbaI fragment, separated in a low melting point agarose gel and extracted using the Geneclean kit. BamHI-XbaI digested pALTERex-1 plasmid was phenol/chloroform extracted and ethanol precipitated. pAlter-IGFBP-2 resulting from ligation was transformed into JM109 cells, and grown on tetracycline plates for 48h at 37°C. Single colonies were picked and grown in LB-tet medium. Mini and maxi preps were prepared (pALTERex1-IGFBP-2 stock solution was present at 436μg/ml in TE buffer)

Mutagenetic primers. For the construction of either P20A or P22A mutations, 2 base pairs of the wt DNA IGFBP-2 sequence were substituted. Mutagenic oligos contained 15 nucleotides of flanking sequence at either side of the 2 base mismatch to allow sufficient hybridisation to the IGFBP-2 sequences. The mutagenic oligos were designed in such a way as to create SacI or PvuII restriction endonuclease sites for P20A and P22A respectively. 33-mer mutagenic oligonucleotides P20A (IGFBP-2) primer GGG CGC GTC GGG TGG AGC TCC GCA GGC GGC CAG and P22A (IGFBP-2) primer GGC GCA GGG CGC GTC AGC TGG GGG TCC GCA GGC(altered bases shown in blue) were supplied, lyophilised from Cruachem Ltd (Glasgow, UK). Each oligonucleotide was resuspended in H_2O at 1μg/ml.

5'phosphorylation of oligonucleotides. 1μg (1μl) of each oligonucleotide was incubated at 37°C for 30min in the presence of 2.5μl of kinase buffer x10, 0.5U (0.5μl)T_4 polynucleotide kinase, 2.5μl of

10mM ATP (in a final volume of 25µl). Kinase was then heat inactivated at 70°C for 10min and the reactions stored at –20°C.

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

Annealing reaction. 10µl of alkaline denatured ds DNA template, 1µl Amp-repair Oligo, 1µl Tet-knockout Oligo, 1µl of phosphorylated mutagenic nucleotides (P20A and P22A) diluted 1:3, 2µl of manufacture's x10 annealing buffer to a final volume of 20µl, were incubated for 5min at 75°C in a water bath. The water bath was then switched off until the temperature reached 45°C (approximately 30min) and finally tubes were put on ice until RT was reached. The following were added on ice: 3µl synthesis x10 buffer, 1µl (5-10units) T_4 DNA polymerase, 1µl (1-3units) T_4 DNA ligase in this order and finally H_2O to 30 µl. Tubes were then incubated for 90min at 37°C.

Transformation of ES1301 mutant competent cells. ES1301(*lac*Z53, *mut*S201::Tn5, *thy*A36, *rha*-5, *metB*1, *deoC*, IN (*rrn*D-*rrn*E) is a mismatch repair minus strain of *E.coli*. High efficiency competent ES1301 cells were provided with the Promega Ultrasites-II kit. For transformation 1.5µl of P20A or P22A mutagenesis reaction (approximately 10ng of template DNA) was used and the general protocol described earlier was followed, except that after the addition of DNA cells were incubated on ice for only 10min. After the cells had recovered at 37°C for 30min with shaking, 500µl of each culture was transferred to a fresh tube containing 4.5ml LB-Amp medium and grown overnight at 37°C with shaking.

Plasmid mini prep. Miniprep preparation, phenol/chloroform extraction and ethanol precipitation of DNA were undertaken following the protocol described above.

Transforming P20A and P22A IGFBP-2 mutant DNAs into JM109 cells. The yield of P20A and P22A mutated DNAs obtained from ES1310 mini prep preparations was estimated on a 1% agarose gel. 5-

10ng of DNA was transformed into JM109 competent cells and the cells were grown overnight on LB-Amp plates at 37°C. Minipreps were prepared and the presence of P20A and P22A mutations was verified by restriction digestion with SacI or PvuII respectively. Resulting fragments were separated on electrophoresis and the result is shown in Fig *3.1.1.*

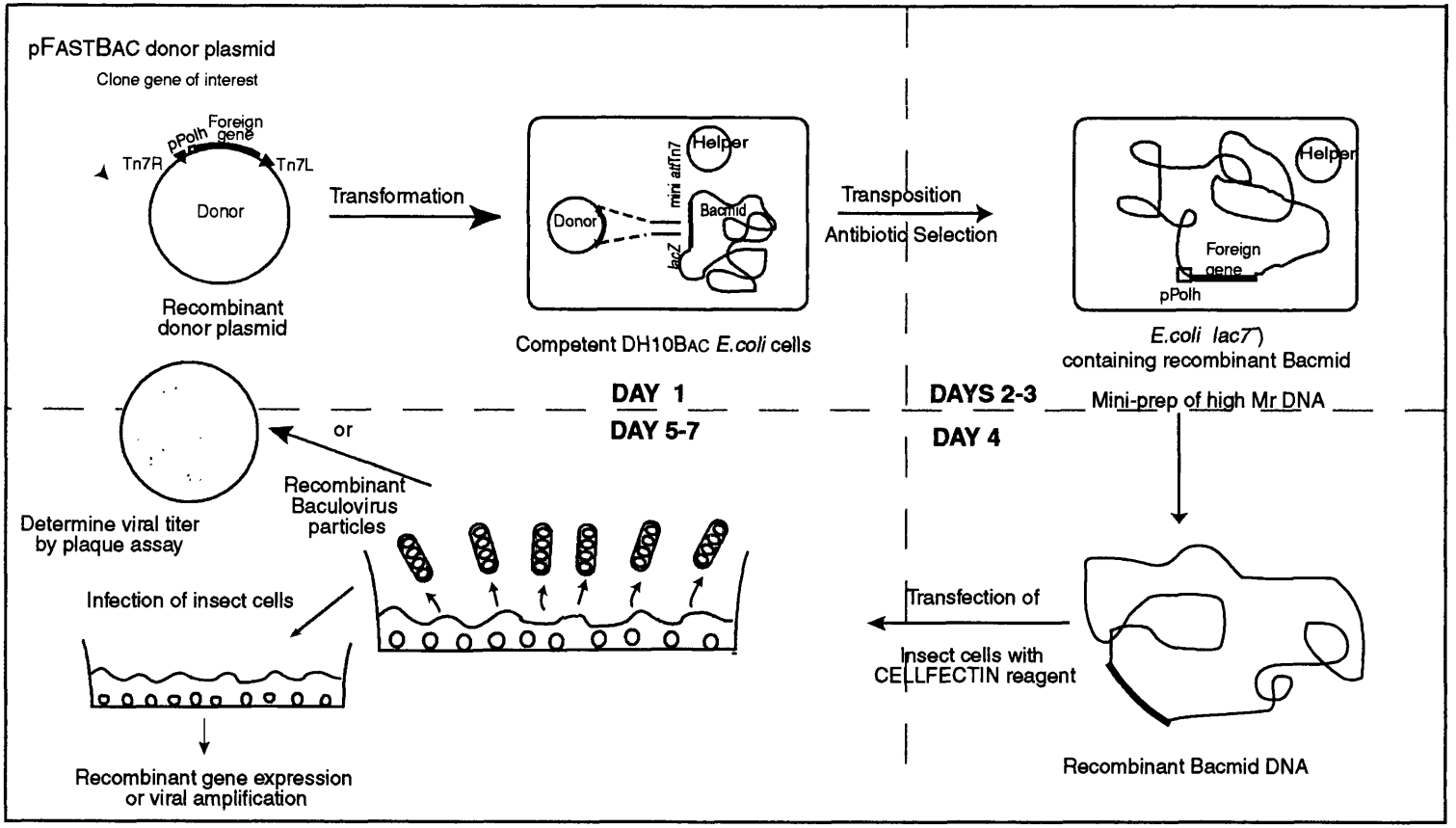
2.1.2 Expression of wt, P20A and P22A IGFBP-2 mutated proteins

We expressed IGFBP-2, P20A and P22A proteins with the Bac-to-Bac™ baculovirus expression system (Life Technologies). In this system the gene to be expressed is cloned into baculovirus and the recombinant virus is used to infect insect cells which express the recombinant protein.

An overview of the system is shown in Fig *2.1.2*

Wt and mutant IGFBP-2 genes were cloned into the donor plasmid, pFastBac, down stream of the polyhedrin promoter of AcNPV (Autographica californica nuclear polyhedrosis virus) baculovirus. In nature, the polyhedrin promoter is activated in the very late phase of baculovirus infection of insect cells (occluded cycle). In this phase virus DNA particles are packaged in polyhedrin protein and stored as occlusion bodies. However, the expression of polyhedrin protein is not essential under *in vitro* conditions where the virus can replicate and infect neighbouring cells even if the polyhedrin gene is substituted with a heterologous gene. In pFastBac donor plasmid, the polyhedrin promoter and the gene of interest are located in an expression cassette flanked by the right and left arm of bacterial transposon gene, Tn7. When pFastBac is transformed into *E. coli* DH10Bac cells, a helper plasmid (pMON7124) provides the functions necessary to transpose the gene of interest from pFastBac to a baculovirus shuttle vector, (the bacmid, pMON14272), contained in the cells. Routinely bacmid can be propagated in *E.coli* DH10Bac cells as a large, low copy number plasmid which confers on the cells kanamycin resistance and the ability to ferment lactose. The bacmid possesses a gene encoding for *lacZ*α peptide which compensates the for *lacZ* deletion in the bacterial chromosome. Therefore, on BluoGal-IPTG plates, DH10Bac colonies appear blue. Moreover the bacmid has inserted inside lacZα gene, a mini-*att*Tn7attachment site for Tn7 bacterial transposon. Inside DH10Bac cells the mini-Tn7 element of pFastBac (including the gene of interest and the protein expression cassette) is transposed at the bacmid mini-*att*Tn7 site. The insertion of the mini-Tn7 element into the bacmid causes the disruption of the reading frame of lacZα peptide. Therefore on

Fig. *2.1.2* Overview of Baculovirus expression system



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

BluoGal-IPTG plates, colonies containing recombinant bacmid will appear white, allowing an easy selection from the background of blue colonies containing non recombinant bacmid.

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

Cloning wt IGFBP-2, P20A and P22A into pFastBac. IGFBP-2, P20A and P22A inserts were obtained by digesting approximately 20μg of pALTER recombinant vectors with 4μl (=40units) of EcoR1 at 37°C 2 h in a final volume of 60μl with appropriate 1x diluted buffer. DNA inserts were separated in a 1% low melting point agarose gel and extracted with the Gene clean kit.

Analogously, 20μg of pFastBac plasmid were prepared for the ligation reaction by digestion with 1.5μl EcoR1 with subsequent dephosphorylation, phenol-chloroform purification and ethanol precipitation. Relative amounts of pFastBac, wtIGFBP-2, P20A and P22A DNAs were estimated on a 1% agarose gel, and 2μg of pFastBac were ligated to 2μg of either wt or mutated IGFBP-2 fragments following the general protocol described earlier. Ligation products were transformed into JM109 cells under ampicillin (100μg/ml) selection. Minipreps of the DNA were prepared and an EcoR1 digestion was performed to detect the recombinant clones. Orientation of the recombinants was verified by a HindIII restriction enzyme digest; HindIII cuts in the polylinker sequence of pFASTBac and at one end of IGFBP-2. Maxi prep DNA was then prepared for each one of the mutants or wt IGFBP-2 proteins.

Transposition. LB agar plates (5g peptone, 2.5g yeast extract, 5g NaCl, 6g agar in 500ml H_2O, pH 7.0) supplemented with 50μg/ml kanamycin, 7μg/ml gentamycin, 10μg/ml tetracycline, 100μg/ml Bluo-Gal and 40μg/ml IPTG, were prepared and stored in the dark at 4°C for a maximum of 2 weeks. DH10Bac cells were transformed with 1ng of wt IGFBP-2, P20A or P22A DNA. The general protocol was followed, with the exception that after transformation 900μl of SOC, instead of LB, medium was added and that the cells were recovered for 4 h at 37°C with shaking. Each culture of transformed cells was serially diluted with SOC medium (10^{-1}, 10^{-2} ,10^{-3}) and 100μl of each dilution was plated onto Bluo-Gal/IPTG plates under drug selection (kanamcin, gentamycin, tertacycline). After 24h incubation at 37°C, 10 large white colonies within a background of small blue colonies were picked, and to confirm the white phenotype, they were re-plated on fresh plates.

Confirmation by PCR that white colonies contained DNA inserts of the correct size. The PCR mix solution was prepared as follows: 15µl M13Reverse primer, 15µl Forward primer, 75µl 25mM $MgCl_2$, 75µl x10 buffer (500mM KCl, 100mM Tris-HCl pH 9, 1% TritonX-100), 5µl dNTP (25mM), 37.5µl DMSO, 527.5µl H_2O and aliquoted into 25µl/tube. The universal primers, forward and reverse, were synthesised by Cruachem. They were received lyophilized and resuspended at 1µg/µl in H_2O A single white colony was swirled in the tube. As negative controls, a blue colony, two minus DNA samples and pFastBac-IGFBP-2 were included. Finally, 0.5µl TAQ polymerase was added and the PCR reaction was performed as follows (94°Cx2min) x1cycle; (94°Cx45sec, 62°Cx45sec, 72°Cx1min) x30 cycles and (72°Cx5min) x1cycle. The presence of inserts was detected by running PCR products in a 1% agarose gel. With the pUC/M13 amplification primers (that are directed at sequences on either side of mini –att Tn7 site within the Lac Z α gene) the expected sizes of PCR products were: Bacmid alone ≈ 300 bps, bacmid transposed with pFastBac ≈ 2300 bps. From blue colonies a 300bps product was obtained, while recombinant bacmids, obtained from white colonies on IGFBP-2, P20A or P22A plates, migrated between the 2300 and 4361 size markers, indicating the presence of the inserts (2300bp of bacmid transposed with pFastBac + ≈ 1000bp of wt or mutated IGFBP-2 inserts).

High Mr plasmid (Bacmid)mini preps. From each of the wt IGFBP-2, P20A, P22A DH_{10}Bac plates a single colony with confirmed white phenotype was picked and cultured at 37°C for 24 h in 2ml of LB medium supplemented with kanamycin, gentamycin and tetracycline (at the same concentration reported above for the plates). High Mr bacmid mini preps were prepared following the protocol recommended by Life Technologies. Briefly: pellets of bacterial cultures were gently resuspended in 300µl of solution I (15mM Tris-HCl, 10mM EDTA pH 8.0, 100µg/ml RNase A). 300µl solution II (0.2N NaOH, 1% SDS) was added and tubes were gently mixed. After a 5min incubation at RT, 300µl of 3M potassium acetate (pH5.5) was added and tubes were incubated on ice for 10min. After a 10min centrifugation at 13000rpm, supernatants were carefully transferred to fresh tubes, which already contained 0.8ml of isopropanol and these were mixed by inversion and incubated on ice for 10min. After a 15min centrifugation at 13000rpm, the supernatants were carefully aspirated, the pellets were washed with 0.5ml 70% ethanol and centrifugation was repeated for 5min as before. Supernatants were carefully aspirated and the pellets air dried. In order to avoid damage to the high molecular weight (Mr) DNA,

pellets were resuspended in 40µl of TE buffer by keeping the tubes on ice for 10min, and flicking them very gently every 2 min. 5µl aliquots for each recombinant Bacmid were stored at –20°C.

Transfection of Sf9 cells with recombinant Bacmid DNA. Sf9 insect cells (>97% viable) were grown in suspension to mid log phase. Cells were then resuspended at $4.5x10^5$ cells/ml in SF900II medium containing 50U/ml penicillin and 50µg/ml streptomycin. 2ml of cell suspension was placed in each well of a 6well plate and cells were left to attach for 1hr at RT. In a tube, 100µl of SF900II medium without antibiotics was mixed with 5µl of each recombinant Bacmid (i.e. carrying either IGFBP-2, P20A or P22A DNA). At the same time 100µl of SF900II medium without antibiotics was mixed with 6µl of Celfectin (Life Technologies) and then added to recombinant bacmid and incubated at RT for 25min. Wells containing cell monolayers were washed with 2 ml of SF900II without antibiotics. Transfection was performed in the following order: i) 0.8ml of SF900 II was added to each tube containing 200µl of lipid-DNA complexes and the contents mixed. ii) Following complete removal of wash medium from one well, the cell monolayer was overlaid with 1ml of transfection mixture and the incubation was left for 5h at 27°C. The transfection mixture was then removed and 2ml of SF900II medium with antibiotics was added, and the plate was incubated for 72h at 27°C. After this incubation period, the supernatants containing recombinant viruses were harvested. Supernatants from 2 wells were pooled, centrifuged at 1000rpm for 5min, transferred to a 15ml tube wrapped with foil and stored at 4°C. 100µl of this sample was assayed for protein expression by Western blot.

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

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

Insect cell culture. The Sf9 insect cell line was originated from pupal ovarian tissue of Spodoptera frugiperda (armyworm) and it has been selected for its high susceptibility to Baculovirus infection. We received as a gift from Life Technologies a frozen vial of cells already adapted to serum free conditions. We expressed IGFBP-2 in Sf9 cells in serum free medium (SFM), protein free (SF900-II Gibco) conditions for several reasons: i) as IGFBP-2 is a secreted protein, we intended to recover the expressed protein directly from the supernatant of a culture of insect cells grown in suspension. The manufacturing company (Life Technologies) that commercialises the Bac-to-Bac expression system claims that, in SF conditions, the recombinant secreted protein represents the major protein species in the insect cell supernatant, so that further purification might not be required; ii) serum bovine IGFBPs would contaminate the recombinant protein preparation and the separation between rat and bovine IGFBPs would require a laborious reverse-phase chromatography step; iii) protease activity contained in the serum could potentially degrade the expressed protein.

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

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

of fresh and cell-conditioned SF900-II medium. Vials were wrapped in cotton wool, placed in a polystyrene box, stored at –70°C overnight and then transferred to liquid nitrogen.

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

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

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

Insect cells were separated from the media by centrifugation at 3000rpm for 2min, supernatants were concentrated on Millipore tubes (Ultrafree-20) by centrifugation at 1700xg for 2hr at 4°C, and stored at –70°C.

2.1.3. IGF-affinity studies on the wt, P20A and P22A IGFBP-2 proteins

IGF peptides

Recombinant human IGF-I and II (animal/media grade) and Des(1-3)IGF-I (receptor grade) were obtained from GroPep Pty Ltd (Adelaide, Australia). Peptides were received lyophilized and were resuspended at a concentration of 1mg/ml in 10mM acetic acid, aliquoted, snap frozen and stored at –20°C.

Radionucleotides

Iodinated IGFs and IGFBP-2 were kindly provided by Dr J.Beattie. 2.5μg IGF-I, IGF-II or IGFBP-2 were iodinated to a specific activity of 50-100μCi/ug as described in Borromeo *et al*, 1996.

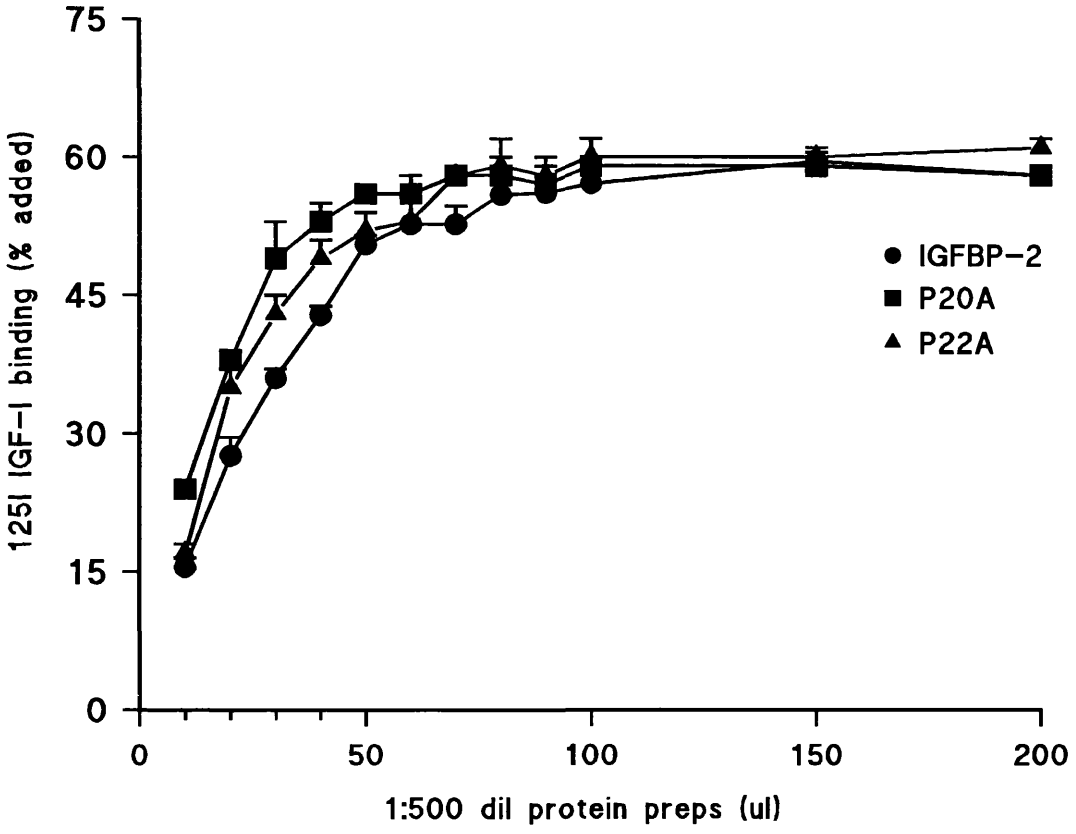
Charcoal binding assay

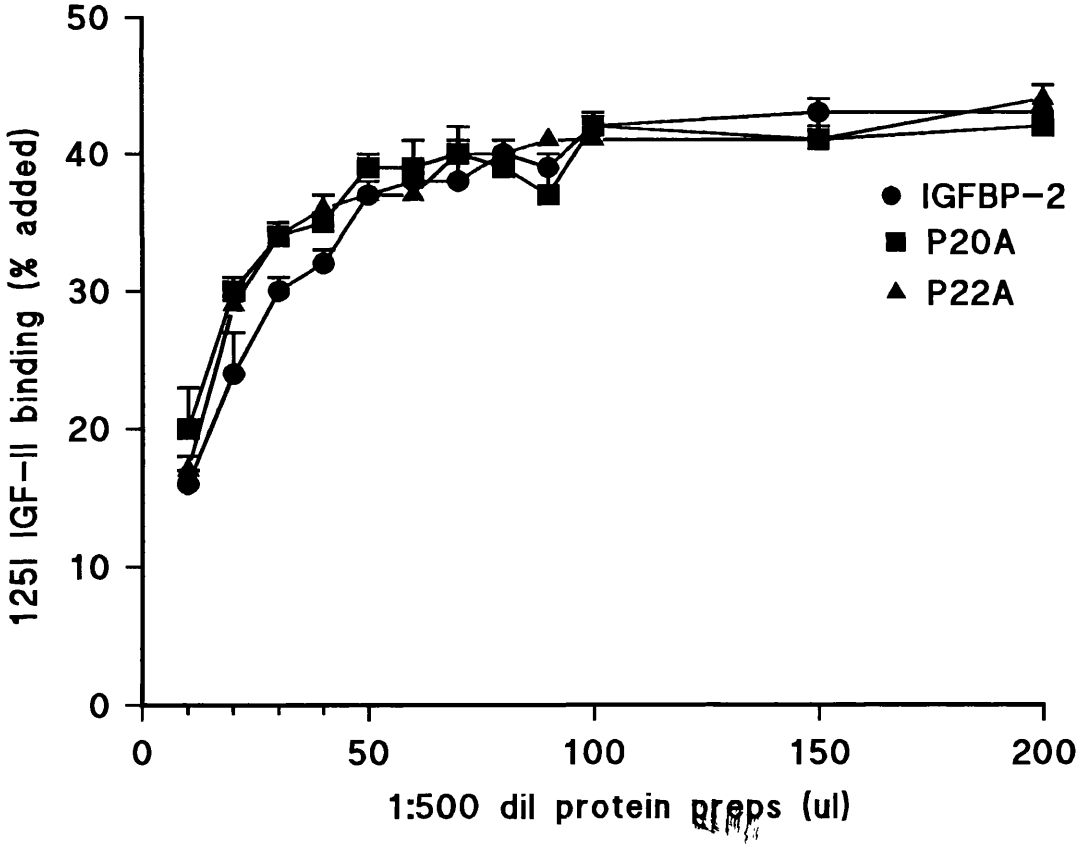
This assay was carried out as in Conover (1989). Typically, triplicate IGFBP samples were added to RIA tubes and appropriate volumes of assay buffer (50mM Tris-HCl, 0.5% BSA, pH7.4) were added to

make the volume up to 100µl. An additional 100µl of assay buffer was then added. If competitive studies were to be undertaken, the latter 100µl of buffer would contain cold IGF-I or IGF-II, at various concentrations. After the addition of further 100µl of assay buffer containing 25000cpm of ^{125}I IGF-I or IGF-II tracer (giving a total volume of 300µl/tube), samples were vortexed and incubated overnight at 4°C. The charcoal buffer (1% activated, neutralized charcoal, 0.2mg/ml protamine sulphate in ice cold assay buffer) was prepared freshly in considerable excess to allow good mixing of the charcoal in suspension and 600µl was quickly added to each sample tube. Samples were vortexed before and after a 10min incubation at 4°C during which free IGF binds to the charcoal, while IGF-IGFBP complexes remain in solution. To separate free from IGFBP-complexed ^{125}I IGF tracer, samples were centrifuged at 3000rpm (1711xg) for 15min at 4°C and, after immediate decanting of the supernatants, pellets were counted in Packard Auto-gamma^{R} γcounter (Packard Instruments Meriden, CT, USA).

Creating an IGF-I and IGF-II binding curve for wt or IGFBP-2 mutated proteins. In a preliminary experiment we tested the IGF-binding capacity of equal volumes of each protein preparation containing either wt IGFBP-2, P20A, or P22A, and the resulting IGF-I and IGF-II binding curves were plotted. Concentrated baculovirus protein preparations were diluted 1:500 in assay buffer and different volumes of these samples (0,10, 20, 30, 40, 50, 60, 70, 80, 90,100, 150, 200µl) were added to RIA tubes. In each tube the volume was brought up to 200µl with assay buffer. Finally, approximately 25000cpm of ^{125}I IGF-I or II in 100µl assay buffer was added and the assay was completed as described above. The radioactivity of the pellets was measured with a gamma counter. The amount of ^{125}I IGF-I or II that, in the absence of IGFBPs, was bound to charcoal pellets was defined as the **blank** value and counts of all other sample pellets were subtracted from this blank value. ^{125}I IGF-I and ^{125}I IGF-II binding curves were plotted as shown in Fig *2.1.3* **a**, **b**. The X axis indicates the volume of the wt IGFBP-2, P20A, P22A preparations. The Y axis, tracer binding is expressed as % of the blank value (labelled "% added"). For each IGFBP the linear part of the binding curve was identified, where the amount of bound tracer increased linearly with the volume of IGFBP preps. For each protein a 20µl volume of the 1:500 diluted protein preparations was chosen for subsequent competition experiments.

Fig 2.1.3 IGF-I and IGF-II binding curves for wt IGFBP-2, P20A and P22A





2.1.3.1 Competition studies using the charcoal binding assay

Competition studies were established to determine the affinity of wt IGFBP-2, P20A and P22A for IGF-I or IGF-II. Based on the results of the previous experiment (see Fig *2.1.3.***a** and **b**), wt IGFBP-2, P20A or P22A preparations were diluted 1:2500 with assay buffer and, 100µl were added to RIA tubes. 100µl of assay buffer +/-increasing amount of cold IGF-I, IGF-II was added. 1mg/ml IGF-I and II stock solutions in 10mM acetic acid were appropriately diluted to obtain a final concentration of 0.25, 0.5, 1, 2.5, 5, 10, 25, 50ng/ml. Finally, approximately 25000cpm of ^{125}I IGF-I or IGF-II in 100µl of assay buffer were added so that wt IGFBP-2, P20A and P22A proteins were each tested for all combinations of competitive binding (IGF-I v ^{125}I IGF-I; IGF-II v ^{125}I IGF-I; IGF-II v ^{125}I IGF-II and IGF-I v ^{125}I IGF-II). The blank value for each of ^{125}I IGF-I and ^{125}I IGF-II tracers was determined and the radioactivity measured in all other charcoal pellets was subtracted from the appropriate blank as described above. ^{125} I IGF-I/II binding of wt or mutated IGFBP-2 proteins in the absence of cold IGFs was designated the **control** value. The residual tracer binding in the presence of cold IGF competitors was expressed as a percentage of control value (100%).

2.2 IGF-II affinity chromatography purification of wt IGFBP-2 and PGD IGFBP-2 mutant

Column preparation

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

Purification of wt IGFBP-2 and PGD IGFBP-2

Protein purification was performed following the method described by Carr *et al.* (1994). 800µl IGF-II-coupled Sepharose gel, prepared as described above, was equilibrated with 10ml 0.5M NaCl, 50mM Tris pH 6.5. 2- 4ml of concentrated insect cell culture supernatant containing IGFBP-2 or PGD-IGFBP-2 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 0.5M NaCl, 50mM Tris pH 6.5 (the first 1ml of the flow through was retained for analysis). 6 ml of 0.5M acetic acid pH3 was incubated with column gel for 10min before starting the elution. 6x 1ml fractions were collected in Eppendorf tubes already containing 300-360µl 2M Tris base necessary for immediate pH buffering to pH 7.0. Fractions were kept on ice and the column washed with 10ml 0.5NaCl, 50mM Tris pH 6.5. Subsequently, alternate washes with 5ml of 0.1M Tris, 0.5 NaCl, pH 8.5 and 5 ml of 0.1M Na acetate, 0.5M NaCl, pH 4.5 were repeated twice. For storage the column was equilibrated in 50mM Tris, 0.5 M NaCl, 0.01% sodium azide, pH 7.4 and kept at 4°C.

IGFBP-2 or PGD-IGBP-2 fractions eluted from the affinity column were analysed for their total protein content by Coomassie blue or Silver staining gels and the presence of purified proteins was confirmed by anti IGFBP-2 Western and ligand blots (as described below). Measurement of protein concentration was estimated by reading spectrophotometrically the absorbance at 280nm, as commercial protein assay kits were unsuitable due to the high concentration of Tris added to neutralise acidic fractions.

General techniques for protein analysis.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out following the method described by Laemmli (1970). Separating gels contained 7.5% or 12% total acrylamide (2.7% Bisacrylamide cross-linker), 375mM Tris-HCl (pH8.8), 0.1%SDS (w/v). Polymerisation was initiated with ammonium persulphate (Biorad) and Temed. Stacking gels were 4% acrylamide, 125mM Tris HCl (pH6.8), 0.1% SDS. Samples for analysis were mixed 1:1 with 62.5mM Tris-HCl (pH6.8), 0.1% SDS, 10% glycerol and 0.05% bromophenol blue. For reducing conditions 5% 2-mercaptoethanol was included in the sample buffer. Electrophoresis was performed at constant voltage

(150V) on a Biorad mini protean II apparatus in tank buffer consisting of 25mM Tris-HCl, pH 8.3, 192mM glycine, 0.1% SDS.

Coomassie blue staining. This technique was employed to visualise protein bands after electrophoretic separation and to estimate their relative concentration.

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

Silver staining. This is a very sensitive technique which was adopted to detect proteins of low abundance. After electrophoresis, gels were sequentially washed at RT, with gentle agitation, in 100ml of sol.1 (50% methanol, 10% acetic acid, 40% H_2O) for 30min (or overnight), 100ml of sol. 2 (5% methanol, 7% acetic acid, 88% H_2O) for 30min and with sol. 3 (2.5% glutaraldehyde, 97.5% H_2O) for a further 30min. To completely eliminate glutaraldehyde, extensive washing in H_2O (8x 15min) over a period of 2 hours was carried out. Gels were then incubated in DTT 0.5mg/100ml H_2O for 30min and with 100ml of 0.1% $AgNO_3$ for a further 30min. During this last incubation, developing solution (150ml of 3% Na_2CO_3 supplemented with 70µl of 40% formaldehyde) and stop solution (5ml of 2.3M citric acid) were prepared. At the end of the 30min incubation in $AgNO_3$, gels were quickly rinsed with water for 30sec and then incubated with 25ml of developing solution. The solution was discarded when brown colouration developed. A second wash with 25ml of developing solution was repeated for 30seconds. The remaining 100ml of developer was added and development of gels kept under observation. The reaction was stopped by the addition of 5ml stop solution, when brown coloured protein bands were clearly visible over the increasingly dark background. Gels were then rinsed in water and dried between cellophane sheets as described for Coomassie staining.

Electrotransfer. As an alternative to direct gel staining, proteins separated by electrophoresis were transferred from the gel to either Hybond-C extra or Hybond-PVDF (Amersham) membranes. In the latter

case membranes required 10sec pre-wetting in methanol and subsequent 5min rinsing in H_2O before use. Gel sandwiches (fibre pad, filter paper, gel, membrane, filter paper, fibre pad) were assembled under transfer buffer (25mM Tris, 192mM glycine, 20% methanol) in a bath and then located in the Biorad mini transblot apparatus equipped with an ice pack. Protein electrotransfer was performed at a fixed voltage (100V) for 1h, unless otherwise indicated. Blotted proteins were usually visualised by Ponceau staining, as described below. Membranes were then either blocked for ligand or Western blotting for immediate analysis or dried and stored between filter paper in a plastic bag at 4°C, for later analysis. Dried PVDF membranes were moistened for 10sec in methanol before being further processed.

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

^{125}I IGF-I ligand blotting. ^{125}I IGF-I ligand blotting was performed essentially as described by Hossenlopp *et al.* (1986).

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

As an alternative to autoradiographs, dried blots were exposed to a Molecular Dynamics Phospho-imager screen for 2 days at RT and the resulting image would be visualized with the PhosphoImager 445SI software.

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

2.3.Studies on biological features of IGFBP-2

2.3.1.Studies on IGFBP-2 association to the cell surface

Cell lines

The *Clone 9* epithelial cell line was derived from Rat Sprague Dawley liver and is listed in the European collection of cell culture with ECACC code No.88072203. Cells were supplied frozen from the European Collection of animal cell culture (ECACC, Salisbury, Wiltshire, UK). Clone 9 cells were grown at 37°C, 5% CO_2, in 75-225cm^2 plastic flasks containing 20-40ml of complete medium (Ham's F12 medium with 2mM glutamine, 10% foetal bovine serum (FBS), supplemented with 100U/ml Penicillin G and 100μg/ml Streptomycin sulphate). 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 3-7ml of x1Trypsin-EDTA solution (0.5g/mlTrypsin and 0.2g/ml EDTA, obtained by diluting x10 concentrated solution with HBSS). 15-20ml of 10% FBS F12 medium were added to inactivate trypsin, then cells were harvested in 50ml centrifuge tubes and centrifuged at 1000rpm for 5min. Supernatant was discarded and cell pellets resuspended in complete medium. Cells were routinely passaged 1:10 and fed with fresh medium every 3 days after washing with HBSS.

The *MC3T3-E1* osteoblastic cell line was a kind gift of Dr. Simon Butterwith (Roslin Institute, Edinburgh)

MC3T3-E1 cells, that have a fibroblastic phenotype, were derived from newborn mouse calvarial cells by Sudo *et al.* (1983). Cells were routinely cultured at 37°C in 10 % new born calf serum (NCS) DMEM(41965-039 Life Technologies) supplemented with penicillin and streptomycin, as described above. Cells were passaged at confluence as for Clone 9 cells.

The *A10* smooth muscle cell line (available in ECACC No CRL1476), derived from rat thoracic aorta, was a kind gift of Dr C. Delves (Glaxo-Wellcome, Stevenage, UK). A10 smooth muscle cells were routinely cultured in DMEM supplemented with 20% heat inactivated (56°C for 30 min) FBS and antibiotics, as described above. Cells were passaged 1:10 as described for Clone 9 cells.

The *3T3F442A* preadipocyte cell line was purchased from Dr Howard Green at Harvard Medical School, Boston, MA USA. Cells were routinely cultured in DMEM 10% NCS in the presence of penicillin and streptomycin and incubated at 37°C at 10% CO_2. If grown in this medium and passaged when subconfluent, 3T3F442A cells maintain an undifferentiated, fibroblastic phenotype. As at confluence confluent cells tends to detach from the plastic surface and as cells appeared to be very sensitive to dehydration, care was taken to carry out experiments at 70-80% cell confluence and to promptly replace medium or washing solutions.

The *Dx3* human melanoma cell line was a kind gift of Dr C. Delves (Glaxo-Wellcome, Stevenage, UK). Cells were routinely cultured in DMEM supplemented with 10% heat inactivated FBS (56°C for 30min) penicillin and streptomycin. At confluence cells were passaged 1:10 by washing with PBS first and then incubating for 1min at 37°C with one part of trypsin and 4 parts of Versene (Life Technologies)

The *K562* human erythroleukemia cells were a kind gift of Dr C. Delves (Glaxo-Wellcome, Stevenage, UK). These cells were grown in suspension in 50% RPMI-50%DMEM supplemented with 10% FBS and antibiotics.

Antibodies:

Polyclonal, rabbit, *anti bovine IGFBP-2* antibody was from Upstate biotechnology (supplied by TCS Biologicals Ltd, Buckingham UK). Obtained from the same source were i) rabbit polyclonal *anti-rat IRS-I* (Pleckstrin homology domain), and ii) *4G10 anti-phosphotyrosine* monoclonal antibody.

Anti-rIGFBP-5, sheep polyclonal antiserum was a gift from Dr E. Tonner, Hannah Research Institute, Ayr, UK.

Anti-h IGF-II polyclonal antiserum (rabbit) was from GroPep.

Anti-hIGF-I was a gift from NIDDK (Bethesda Maryland, USA).

Enzyme-conjugated antibodies, anti-rabbit, anti-sheep or anti-mouse *HRP-conjugated*, were from Sigma.

2.3.1.a Screening of different cell types and different methods to detect the association of exogenously added rat recombinant IGFBP-2 with the cell surface.

2.3.1.a.1 Use of an immunoistochemical technique (anti IGFBP-2) to detect recombinant rIGFBP-2 or PGD-IGFBP-2 mutant binding to Dx3 cell monolayers

96 well plates (Life Technologies Maxisorp Nunc immuno-plates) were coated with 2μg/well fibronectin or with 2.5μg/well poly L-lysine and incubated at 37°C for 1h (or at 4°C overnight). After washing the wells three times with PBS, Dx3 cells were plated at $1x10^5$ cells/well in 100μl of 1% BSA, 0.1mM Mg^{++} DMEM and allowed to attach for 1hour at 37°C. In order to test the background binding of recombinant IGFBPs to plastic, cells were omitted in some wells. After washing the plate twice with PBS, 0.5-2μg/well of recombinant IGFBP-2 or PGD-IGFBP-2 in 100μl of 1% BSA, 0.1mM Mg^{++}/DMEM was added to cell monolayers or control wells. The binding reaction was allowed to proceed for 1h at 2 different incubation temperatures, 21°C or 4°C, as IGFBPs could be internalised after association with the cell membrane. Cells were washed three times with ice cold PBS, and fixed for 10min on ice with 4% paraformaldehyde, which also has cross-linking properties. After extensive washing, non-specific binding sites were blocked with 1% BSA for 45min at RT. For immune-detection of bound IGFBP, anti IGFBP-2, diluted at 1:2500 in 1% BSA PBS, was added and incubated overnight at 4°C. After 3 washes with PBS,

the second antibody, anti rabbit HRP (Ig HRP-linked $F(Ab)_2$ fragment, code NA9340, Amersham) diluted at 1:20000 in 1% BSA PBS was added and incubation was allowed for 1h at RT. After 5 washes with PBS, the presence of immuno reactive IGFBP-2 proteins was revealed with Sigma Fast™ ODP peroxidase substrate system (code P 9187). One o-phenylenediammine dihydrochloride and one urea hydrogen peroxide tablet were dissolved in 20ml H_2O. 200µl/well of this solution was added, then plates were incubated in the dark for 30min and the colourimetric reaction was measured with the spectrophotometer at 450nm.

2.3.1.a.2. ^{125} *I IGFBP-2 binding to A10 and Clone 9 cell surface in competition with cold IGFBP-2*

Clone 9 and A10 cells were plated on 24 well plates at density of $0.8x10^4$ and $2.2x10^4$ cell/well respectively and allowed to reach confluence. In some cases cells were omitted, but wells were treated exactly in the same way as follows. After washing twice with HBSS, cells were serum-starved in F12 or DMEM containing 0.01% BSA and incubated overnight at 37°C. Supernatants were removed and cell and control wells were washed with ice cold Hepes binding buffer (H.b.buffer): 20mM Hepes, 118mM NaCl, 5mM KCl, 1.2mM $MgSO_4$, 8.8mM dextrose (D glucose), 0.1% fatty acid free BSA, pH 7.4 (as in Yang et al., 1996). 85000cpm of ^{125}I IGFBP-2, dissolved in H.b. buffer, was added to monolayer or control wells in the presence or absence of 0.1µg or 1µg of cold IGFBP-2 (in a total volume of 200µl/well) and incubation was continued for 3h at 8°C. Wells were washed 3 times with ice cold H.b.buffer and residual radioactivity was removed from monolayers and control wells with 500µl 1N NaOH. After monitoring cell lysis microscopically, supernatants were transferred to RIA tubes and radioactivity determined by γ-counting.

2.3.1.a.3 Stripping endogenous IGFBP-2 potentially bound to the cell membrane before incubating cells with ^{125}*I IGFBP-2.*

Clone 9 and A10 cells were plated in 6 well plates and grown with serum containing medium till confluence. Some of the control wells did not contain cells, but they were treated identically. After washing with HBSS, cells were incubated for 24h in SFM containing 0.01%BSA. Stripping of endogenous IGFBP potentially associated to cell membranes was performed as in Andress (1995). After

washing with SFM, wells were sequentially rinsed with i) 2ml of ice cold 2M NaCl, 20mM Na acetate, pH4.0, ii) 2ml ice cold 2M NaCl, 20mM Hepes, pH 7.5 and iii) PBS. Cell monolayers were then incubated for 2h at 4°C with 150000cpm ^{125}I IGFBP-2 dissolved in 20mM Hepes, 0.1% BSA, pH7.0 in the presence or in the absence of 1μg of cold IGFBP-2. After rinsing the wells three times with ice cold PBS, residual radioactivity was extracted with 500μl/well 1N NaOH shaking for 1h at RT and radioactivity detected by γ-counting.

2.3.1.a.4. ^{125}I IGFBP-2 binding to cell monolayers cultured on positively charged plates

In our attempt to minimise ^{125}I IGFBP-2 non specific binding to plastic, Clone 9 cells were cultured on positively charged 6 well plates (Primaria^{R} plates code 3846 Becton Dickinson labware supplied by Fred Baker Scientific, Cheshire,UK) and grown until they became confluent. Monolayer and cell-free control wells were washed 3 times with PBS and incubated for 4h at RT with 500μl Hepes b.buffer containing 70000cpm of ^{125} I IGFBP-2 in the presence or absence of 25μg of cold IGFBP-2. After washing, 500μl 1N NaOH/well was added and plates were shaken for 10min. Supernatants were transferred to RIA tubes and radioactivity determined by γ-counting..

2.3.1.a.5 ^{125}I IGFBP-2 binding to cells in suspension

In an attempt to decrease ^{125}I IGFBP-2 non specific binding to plastic, the cell membrane/plastic surface ratio was increased by using a high density cell suspension placed in RIA tubes. A10 and 3T3F442A cells were plated in 10cm dishes and grown at 37°C till 80-90% confluence. Dishes were washed twice with cold SFM, scraped into 5ml SFM and sequentially passed through an 18G and 23G needle. Cell suspensions were washed in H.b. buffer, centrifuged at 1700xg for 5min, resuspended at 1x10^{6} cells/100μl H.b buffer and placed in RIA tubes. A10 cells were incubated with 6000 cpm of ^{125}I IGFBP-2 in the presence or absence of 0.1or 1μg/ml cold IGFBP-2 (in a total volume of 300μl H.b.buffer). 3T3F442A cells were incubated with 160000cpm of ^{125}I IGFBP-2 and cold competitor was added at 0.3μg/ml or 3μg/ml. During the 3h incubation at 8°C, cell suspensions were mixed from time to time. Tubes were centrifuged at 1700xg for 10min at 4°C and radioactivity in both pellets and supernatants was determined by γ-counting.

2.3.1.a.6 ^{125}I IGFBP-2 ligand blots on Clone 9 and MC3T3-E1 cell membrane preps.

Clone 9 and MC3T3-E1 membranes were prepared as described in paragraph 2.3.1.b. Membrane samples were electrophoresed, blotted and processed for ligand blotting as described in paragraph 2.2 with the exception that, instead of IGF-I, ^{125}I IGFBP-2 was used as probe for detection of a possible binding partner present in membrane preparations.

2.3.1.a.7 ^{125}I IGFBP-2 cross-linking to Clone 9 membranes treated (+/-) sodium chlorate.

Clone 9 cells were plated onto 10cm dishes and grown to confluence in the presence or absence of 30mM Na chlorate. Plasma membranes prepared from treated and untreated cells were cross-linked with ^{125}I IGFBP-2 following the method described in paragraph *2.3.1.b.1*

2.3.1.a.8 Preliminary study to detect exogenous/endogenous IGFBP-2 binding to Clone 9 cell extracellular matrix..

ECM preparation was obtained following the method described by Jones *et al.*, 1993. Clone 9 and MC3T3-E1 cells were plated on positively charged Primaria^{R} 6 well plates (described above) and grown at 37°C. When confluence was reached, wells were washed twice with ice cold PBS. Cell membranes were dissolved by shaking the plates for 10min on ice with 500µl/well of 0.5% Triton X-100 in PBS, pH7.4 and removed. Nuclei and cytoskeleton elements were then removed with 500µl/well of 25mM NH_4 acetate pH9.0 shaking the plates on ice for 10min. The residual ECM-coated plates were used immediately. 70000cpm ^{125}I IGFBP-2, in 500µl Hepes binding buffer, were added to ECM coated or cell-free control wells and incubated for 48h at 4°C. Supernatants were then aspirated and wells washed three times with PBS. Residual radioactivity was removed either with 1N NaOH or 2% SDS and determined by γ-counting. Alternatively samples from each step of ECM preparation described above were electrophoresed on a 12% gel, blotted and processed for either ^{125}I IGF-I or ^{125}I IGFBP-2 ligand blot. In this last case half of the blot was incubated with ^{125}I IGFBP-2 tracer in the presence of 25µg cold IGFBP-2.

2.3.1.a.9.a Integrin studies: Fibronectin binding assay on K562 cells using IGFBP-1 as competitor

96 well/plates (Maxisorp) were coated with 2µg/well fibronectin as described in 2.3.1.a.1. After washing in HBSS 0.5mM $MnCl_2$, K562 cells were resuspended in the same medium at a density of $6x10^5$

cells/ml. 100µl of cell suspension was incubated for 3hr at RT in the presence of either 2µg IGFBP-1(Sigma); 2µg (IGF-I-IGFBP-1 complex), 20µg RGD, 20µg RGE, 1,2,5,10,20µg 4B4 Ab; 10,20µg mouse IgG_1 Isotype Ab or PBS. For each condition tested, 50µl/well of cell suspension were plated in duplicate onto fibronectin plates. Cells were left to adhere for 30min at 37°C, washed twice with PBS and fixed with 100% ethanol for 30min. After cell staining with 0.1% crystal violet for 1h at RT, plates were rinsed with tap water and lysed with 50µl of 0.5% Triton and shaken for 10min. 150µl of water was added and, after mixing, the absorbance was read at 570nm

2.3.1.a.9.b Integrin studies: cell adhesion test on IGFBP-2 and IGFBP-1 coated plates

After trypsinisation, Dx3 cells were resuspended at $1x10^6$cells/ml in DMEM in the presence or absence of 1mM Mg^{++}. 100µl of cell suspension was plated on Maxisorp plates coated with 10-50-100-500µg/well of IGFBP-2 or non phosphorylated IGFBP-1 (Sigma) and allowed to attach for 1hour at 37°C or 2hr at 4°C. After careful washing with PBS cells were fixed with 37% formaldehyde for 30min at 4°C. After washing with PBS for 5min, cells were stained with crystal violet and lysed as described above. Absorbance was determined at 570nm.

2.3.1.b Detection of endogenous IGFBP-2 associated to the cell surface.

Cell membrane preparation

Cell membranes were prepared from cell monolayers as in Woodward *et al.* (1996). Typically cells would be plated on 20x10cm Petri dishes in complete medium and incubated at 37°C till confluence. After washing twice with 10 and 5ml of HBSS, cells were grown overnight at 37°C in 5ml of serum free medium (which has the same composition as complete medium, except serum was substituted with 0.01% BSA). The following procedures were all carried out on ice and using ice cold solutions. After washing twice with PBS and rinsing quickly with 5ml of hypotonic buffer (1mM EDTA, 1mM $NaHCO_3$, pH7.4), cell plates were incubated for 10-20min with 3ml/plate of hypotonic buffer supplemented with proteases inhibitors (2mM PMSF, 3uM leupeptin, 16uM aprotinin, 500mM $NaVO_4$). Cells were scraped from all plates, pooled and kept on ice. Residual cells were harvested by washing each plate with 2ml hypotonic buffer (with protease inhibitors) which were pooled with the rest of cell lysate and then

homogenized for 10sec at half maximal speed with a Polytron homgenizer (Kinematica, Luzern Switzerland). A 10min centrifugation at 1000xg at 4°C removed the nuclear fraction. Supernatant was transferred to 2x30ml tubes (Nalgene, Rochester, NY, USA) and centrifuged at 15000xg (13000rpm in SS34 rotor) at 4°C for 10min to remove lysosomal contamination. This supernatant was transferred to 50ml Sorval tubes and ultracentrifuged at 50000xg (26000rpm on A641 rotor, Beckman) at 4°C for 1.30h. After careful removal of the supernatant, the small, transparent, lipidic pellet was resuspended in a small volume (200-300µl) of 10mM Tris pH7.4 supplemented with proteases inhibitors (as for hypotonic buffer). Membrane preps were frozen in liquid nitrogen and stored at –20°C. Protein concentrations were assayed on the day of the experiment and the membrane preparation further diluted as required.

2.3.1.b.l ^{125}I IGF cross-linking studies on Clone 9 and MC3T3E1 cell membranes

^{125}I IGF-I and IGF-II cross-linking experiments were carried out adapting the method described by Reeve *et al.* (1993).

400 µg/tube of membranes were resuspended in Tris buffer (5mM Tris pH 7.4 , 0.5% fatty acid free BSA) and, after the addition of cold competitors (1µg IGF-I/II, desIGF-I, or 10µg insulin), the volume was adjusted to 500µl. An additional 500µl of Tris buffer containing 1x10^6 cpm of ^{125}I IGF-I or IGF-II was added to each tube followed by a 3hr incubation at RT with rotation. Membranes were pelleted at 14000rpm at 4°C for 10min, and after aspirating radioactive supernatant, the pellet was counted and washed with 1ml of Tris buffer. Centrifugation was repeated, supernatants discarded and, after recording the c.p.m., pellets were resuspended in 1ml of ice cold Tris buffer (without BSA) for the cross-linking reaction. 40mM Disucciminidyl suberate (DSS) in 100% DMSO anhydrous was prepared freshly and kept at RT 50µl of this solution was added to each membrane sample and incubated on ice for 15min. The cross-linking reaction was quenched by the addition of 200µl of stop solution (50mM Tris pH 7.4, 5mM EDTA) and tubes were centrifuged at 14000rpm for 10min at 4°C. Radioactive supernatants were separated carefully (after recording supernatant and pellet counts), and cross-linked pellets were resuspended in 2x reducing SB, boiled for 3min and run in 12% or 7.5% acrylamide SDS gel.

A recombinant rIGFBP-2 sample was included in the cross-liking reaction as control. Our preparation of IGF-II affinity chromatography purified IGFBP-2 contained high concentrations of Tris-base used to

neutralize the pH. Primary amines found in Tris would interfere with the DSS cross-linking reaction, therefore we drop-dialysed 10-20 µl of 170µg/ml IGFBP-2 on 0.025µ Millipore membrane disks (cat VSW 02500) against 70ml water for 15min and replaced once for a further 15min. 10µl of dialysed IGFBP-2 was incubated with 50µl of IGF tracer (dissolved in 5mM Tris pH7.4, 0.5% fatty acid BSA as described above) and rotated for 3h at RT. The cross-linking reaction was performed by adding 2.5µl of DSS solution directly to the tubes (containing 0.5% BSA). After 15min incubation on ice, the reaction was quenched with 10µl of stop solution and the white pellet resuspended. 20µl of this suspension were mixed with 20µl of reducing 2x SB.

2.3.1.b.2 Detection of IGFBP-2 associated to Clone 9 membranes by ^{125}I IGF-I ligand blot and anti IGFBP-2 Western blot.

Crude preparations of Clone 9 cells were electrophoresed on a 12%acrylamide gel under non reducing conditions and after transfer blotting, membranes were processed for ^{125}I IGF-I ligand blotting or Western blotting according to the general procedure described in paragraph 2.2. For anti IGFBP-2 Western blotting, the first antibody (rabbit anti bovine IGFBP-2) was diluted 1:2000 and anti rabbit HRP-conjugate second antibody was diluted at 1:5000. Incubation and dilution buffers have been described earlier.

Sheep anti rIGFBP-5 immune serum (1:1000) was used as negative control in Western blotting experiments (data not shown). In this case second antibody was 1:20000 dilution of anti-sheep HRP-conjugate antibody (Sigma).

2.3.1.b.3 Anti IGFBP-2 and 5 immune-precipitation (IP) of solubilized Clone 9 and MC3T3–E1 membrane preps. Detection of precipitated proteins by ^{125}I IGF-I ligand blot.

Buffer A (50mM Tris-HCl pH 7.4, 150mM NaCl, 0.01% NaN_3) and buffer B (20mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 0.2% BSA) were prepared.

800µg of Clone 9 or MC3T3-E1 membranes were resuspended in a final volume of 400µl of buffer A supplemented with protease inhibitors (0.8mg/ml pepstatin, 2mg/ml aprotinin, 2mg/ml leupeptin,) and with detergents (1% Triton-X100, 1% Na-Deoxycholate). Membrane samples were allowed to solubilize by rotating at 4°C for 1hr. 400µl of buffer B was added and insoluble debris was removed by

centrifugation at 14000rpm for 30min at 4°C. Supernatants were transferred to fresh tubes and 10μl of anti IGFBP-2 antibody or 10μl of anti IGFBP-5 anti serum were added. To pellet the immune-precipitated complexes, 50μl of Pansorbin were added, tubes were rotated for 1h at 4°C and centrifuged at 6500 rpm for 10min at 4°C. Supernatants were aspirated and pellets were washed 3 times with 1ml buffer A (without protease inhibitors or detergent). Pellets were mixed with 50μl of 2x non reducing SB, incubated for 30min on ice, boiled for 3min, electrophoresed on a 12% gel and finally processed for ^{125} I IGF-I ligand blot.

As a control for the immune-precipitation reaction, i) 10μl of 70μg/ml IGFBP-2 and 10μl of baculovirus expressed, non purified, IGFBP-5 (a kind gift of Dr E. Tonner) were dissolved in 200μl of buffer A in the presence or in the absence of 4μl of anti IGFBP-2 or anti IGFBP-5 antibodies. ii) 4μl of anti IGFBP-2 or anti IGFBP-5 was diluted in 200μl of buffer A in the absence of IGFBPs. 200μl of buffer B was added to all control samples, which were rotated overnight at 4°C. Pansorbin precipitation was performed as described for membrane samples. (Only some of these controls are shown).

2.3.2 Clone 9 cell responsiveness to IGF stimulation.

2.3.2.1. Signalling studies

Clone 9 cells were plated in 10cm dishes in 10% FCS Ham's F12 and incubated at 37°C in 5% CO_2 until confluence was reached. Cells were then washed with HBSS and serum starved overnight in 5ml of 0.01% fatty acid free BSA in Ham's F12. Lysis buffer was prepared fresh on the day of the experiment, by thawing a 20ml aliquot of buffer (50mM Tris HCl pH7.4, 1% NP40, 0.25% Na-deoxycholate, 150mM NaCl, 1mM EDTA, aliquoted in 20ml and stored at –20°C) and adding 200μl $NaVO_4$ (stock solution 100mM in water, stored at –20°C), 20μl leupeptin and 20μl aprotinin (stock solutions 1mg/ml in water, stored at –20°C) and 20μl PMSF (1M solution in 100% Ethanol prepared fresh). Stock solutions of IGF-I, IGF-II (1mg/ml in 10mM acetic acid), des (1-3) IGF-I (0.5mg/ml in water) and insulin 1mg/ml (in 10mM HCl) were diluted in 5ml SFM at concentrations specified for each experiment. Cells were washed with 5ml of pre-warmed HBSS, then 5ml of pre-warmed medium with or without hormones was added and incubated at 37°C for 2-10min as indicated for each experiment. Medium was discarded, and plates placed on ice and immediately rinsed with 10ml of ice cold PBS.

Washing was repeated once and PBS aspirated completely. 300µl of cell lysis buffer was added to each dish and cells scraped and collected in a 1.5ml tube and kept on ice. Cell lysates were rotated end over end for 20min at 4°C, then centrifuged at 10000xg at 4°C for 10min. Supernatants were transferred to fresh tubes and protein concentration determined with BCA protein concentration kit (Pierce).

All samples were normalized by adding an appropriate volume of lysis buffer. A 20µl sample was mixed with 20µl of 2x reducing SB, run in a 7.5% gel and blotted on nitrocellulose membranes. The first antibody, 4G10 anti phopsho-tyrosine monoclonal antibody was diluted 1:1000-1:4000 (as indicated in figure legends) and the second antibody, anti mouse HRP-conjugate, at 1:1000-1:2000. Western blot was performed following the general description as in Methods section *2.2.*

Membranes were also examined by anti IRS-I Western blotting. Anti-IRS-I, (rabbit polyclonal IgG) (Upstate biotechnology cat. N. 06-524), was diluted at 1:1000 in 1% BSA TBS-T. Second antibody, anti rabbit HRP-conjugate was diluted at 1:5000 in 5% skimmed dried milk in TBS-T.

2.3.2.2. MTT assay

This assay is based on the conversion of MTT, a water soluble tetrazolium salt, to water insoluble formazan by mitochondrial dehydrogenases. MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue), added to the cell culture as a yellowish solution, is reduced in mitochondria where it accepts electrons from ubiquinone and cytochromes b and c (Kiernan, 1981). Reduction opens the tetrazolium ring and MTT is converted to a purple insoluble formazan. Solubilization of formazan is obtained with isopropanol and the spectrophotometrical absorbance is a function of the converted dye concentration. The MTT method has been used in place of thymidine uptake, for cell counting, or for measurement of cell cytotoxicity and it monitors both the metabolic and mitogenic activity of cells (Mosmann, 1983).

The cell density to be used in the following experiments was established in preliminary studies.

Clone 9 cells were trypsinized from a 225cm^2 flask, pelletted by centrifugation at 1000 rpm for 5min, washed in 0.1% FCS Ham's F12 and recentrifuged. Cell pellets were resuspended in 10ml of 0.1% FCS Ham's F12 then cells were counted and diluted to 1.2x 10^5cells/ml. In a 96 well plate, 50µl of cell suspension was plated in rows proceeding from well 1 to 12 and coming back from 12 to 1 in the next row, in order to obtain an homogeneous distribution of cells within the columns. Moreover during the

plating procedure, care was taken to mix the cell suspension frequently but gently, by inverting the tube. After plating, cells were incubated at 37°C overnight to allow cell attachment. IGF-I, IGF-II, des (1-3)IGF-I or insulin were diluted in 0.1% FCS Ham's F12 to required concentrations as indicated for each experiment. For each treatment a column of 8 replicate wells was used. 50µl of 0.1% FCS Ham's F12 with or without hormone supplementation was added to each well and cells were incubated for 24 or 48 hours at 37C, 5% CO_2. 10µl of MTT solution (5mg MTT/ml in RPMI without phenol red, filtered and stored at 4°C in the dark) was added and cells were incubated at 37°C for a further 3hr. The water insoluble formazan dye which formed was dissolved with 150µl of 0.05M HCl in isopropanol by thorough trituration. Absorbance was determined at 620 nm .

2.3.2.3 Characterisation of IGF production by Clone 9 cells

4 x 225 cm^2 flasks of confluent Clone 9 cells were washed with HBSS twice and incubated in serum free, protein free Ham's F12 medium for 48h. 100ml of CM was harvested, centrifuged at 3000rpm to eliminate cell debris and the supernatant was freeze dried overnight (Heto freeze drier). Lyophilised medium was dissolved in 5ml of water and dialysed against 10mM phosphate for 48h at 4°C. After dialysis, CM was again freeze dried and resuspended in 1ml of water, giving a 10 fold concentration from the original medium.

Detection of IGFs by western blotting was performed as described by Hizuka *et al.* (1998). 15µl of Clone 9 CM sample, and 10, 100, 1000ng of hIGF-I or hIGF-II were run under non reducing conditions in a 16% acrylamide SDS gel and then transferred as described above. First antibody, either anti IGF-I or anti IGF-II, was diluted at 1:1000 in 1% BSA TBS-T. In both cases, the second antibody was anti rabbit HRP diluted at 1:5000. Buffers, incubation conditions and detection are described elsewhere (Methods section *2.2*).

2.3.3 Development of a cellular model to investigate IGFBP-2 biological functions

2.3.3.1 Cloning IGFBP-2 and IGFBP-5 into the pcDNA3 expression vector, in sense and antisense orientations.

pcDNA 3 plasmid (Invitrogen), a CMV-based mammalian-expression vector was a kind gift from Dr M. Travers.

The cloning of IGFBP-2 and IGFBP-5 cDNAs into pcDNA3 and the restriction digests of the recombinant plasmids to check the orientation of the inserted DNA, were performed following the general procedures described in paragraphs *2.1.a.*and *2.1.1.* Briefly: 2μg of pcDNA3 was digested with EcoR1, treated with alkaline phosphatase (Boehringer) and purified with Quiaquick gel extraction filters. IGFBP-2 and IGFBP-5 cDNA inserts were prepared from 10μg of clones contained in the recombinant pGEM 7Zf (+/-) plasmid by an EcoR1 digestion and purified on a 1% agarose gel. IGFBP-2 and IGFBP-5 inserts were excised from the gel and purified on Quiaquick gel extraction filters. After checking the relative amount of DNA on agarose gel, a 1:1 and 1:10 ratio between EcoR1-digested pcDNA3 and IGFBP-2 or IGFBP-5 inserts were chosen for ligation. Following transformation with the ligation products, JM 109 competent cells were grown on LB-agar plates under ampicillin selection. 10 single colonies from each plate were picked and DNA plasmid minipreps were prepared (with UltraClean mini plasmid prep kit, MoBio laboratories, Solana Beach, Ca). The miniprep DNAs were analyzed on a 1% agarose gel after being digested with EcoR1 to check the size of any released inserts and with Hind III to assess the orientation of the inserts with respect of the cytomegalovirus promoter (Fig *3.3.3.1*). Maxipreps were prepared for either orientation of pcDNA-IGFBP-2 and 5.

2.3.3.2 Preliminary experiments to evaluate the growth rate in Clone 9 cells transfected with sense/antisense IGFBP-2 in comparison to wt cells transfected with vector alone

Transfection of Clone 9 cells with pcDNA3 constructs

We transfected Clone 9 cells with sense/antisense IGFBP-2; sense/antisense IGFBP-5 or pcDNA3 vector alone and created 5 different Clone 9 cell subtypes.

Clone 9 cells were plated in 60mm dishes at $2.5x10^5$cells per dish and grown in complete medium. After 24h incubation at 37°C, the cells reached 50% confluence and were transfected with pcDNA3 vector alone; sense/antisense IGFBP-2 or sense/antisense IGFBP-5. Lipofectin (Life Technologies) was used to promote the transfection, which was performed following the instructions described by the manufacturer. The incubation of cells with the DNA-Lipofectin mix was carried out for 6h, after which the supernatants were removed the cells were allowed to recover for 48h at 37°C with 10% FBS F12 Ham's medium supplemented with penicillin and streptomycin. The cells were then passaged 1:5 to fresh 60mm dishes

and, as pcDNA3 confers neomycin resistance, transfected cells were selected with 400μg/ml of a neomycin analogue, G418 (Geneticin, Life Technologies). Few cells from each transfection survived G418 selection, but after a recovery period they started to proliferate, forming distinct foci. Drug selection was maintained for one month. Subsequently the G418 concentration was reduced to 200μg/ml for 2-3 days , then the drug was omitted for a further 1-2 days and the cells were frozen in liquid nitrogen.

MTT assay on Clone 9 cells transfected with sense or anti sense IGFBP-2 or pcDNA3 vector alone.

Frozen vials of Clone 9 cells, which were transfected with pcDNA3 vector alone, sense or antisense IGFBP-2, were thawed and grown for 2 passages under drug selection (400μg/ml of G418)

The MTT assay was carried out substantially as described in paragraph *2.3.2.2.* For each cell line, 7 plates (21 in total) were prepared. Each cell line was trypsinized, washed and resuspended at $6x10^4$ cell/ml in 0.1% FBS. 100μl/well of each cell suspension were placed in 24 of the 96 wells of a plate. Cells were grown at 37°C, 5%CO_2. After 4h, one plate for each cell line was supplemented with 10μl/well of MTT solution (5mg/ml of MTT in RPMI w/o phenol red). These plates were further incubated for 3h to allow formazan production by the cells. The dye produced was solubilised by the addition of 150μl of isopropanol 0.05M HCl/well and absorbance was determined at 620nm. The remaining plates were identically processed every 12hours, over a period of 72h.

2.3.3.3. ^{125}*I IGF-I charcoal binding assay on CM samples from Clone 9 cells transfected with sense/antisense IGFBP-2 or pcDNA vector alone.*

Clone 9 cells transfected with sense/antisense IGFBP-2 or pcDNA3 expression vector were grown in 10cm dishes in 10% FBS Ham's F12. At confluence the cells were washed with HBSS and grown for 24h in 5ml of SFM. Conditioned media was harvested and the IGF-binding capacity of increasing volumes (from 10 to 200μl) of each CM was assessed in our charcoal binding assay following the general procedure described in Materials and Methods (paragraph *2.1.3*).

3. RESULTS

3.1 Construction of P20A and P22A IGFBP-2 mutants, expression of wt IGFBP-2 and mutated proteins and assessment of their affinity for IGFs

3.1.1 Site-directed mutagenesis to create P20A and P22A IGFBP-2 mutants

We used the Promega "Altered sites" system to mutate the wt rat IGFBP-2 cDNA and create P20A and P22A mutants, which encode proteins where the proline residues, at position 20 or 22 of the mature protein were substituted with alanine residues. For P20A, the triplet CCC, at position 256-258 of rat IGFBP-2 cDNA was substituted with CGT. This modification of the original sequence also created a novel restriction site for the restriction endonuclease SacI.

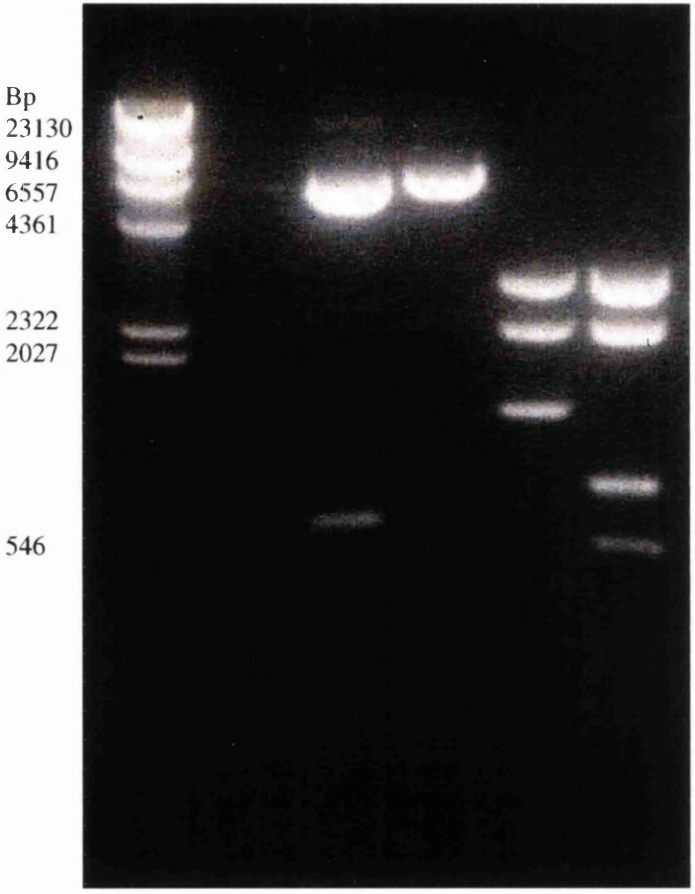
Similarly, for the construction of P22A DNA, the triplet CCC at position 262-264 of rat IGFBP-2 cDNA was replaced with GCT. This modification formed a novel restriction site for the PvuII restriction endonuclease. Fig *3.1.1* shows a 1% agarose gel of wt and mutated IGFBP-2 DNAs (inserted in pALTER ex-1 plasmid) digested with SacI and PvuII endonucleases.

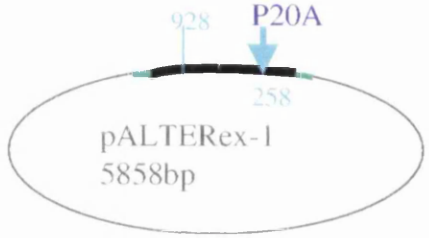
On the same figure two diagrams illustrate the DNA restriction sites for each of the mutants and the expected size of the fragments resulting from endonuclease digestion.

There are no SacI sites in pALTER ex1. One restriction site for SacI, at about position 930 bps, is present in wt IGFBP-2 DNA, therefore digestion with SacI results in a linearization of pALTER-IGFBP-2. As the mutation introduced at the triplet 256-258 of IGFBP-2 DNA for the construction of P20A mutant created a new recognition site for SacI, an additional fragment of about 670 bps was released following the digestion.

There are no PvuII restriction sites in the IGFBP-2 cDNA, but this endonuclease cleaves pALTERex1 plasmid at three sites, demonstrated by the 3 fragments that originated from the digestion of pALTER-IGFBP-2. The mutation of IGFBP-2 DNA at the triplet 262-263 created a novel restriction site for PvuII and, as expected, we saw 4 fragments resulting from digestion with this endonuclease.

Fig *3.1.1* DNA restriction map of P20A and P22A mutants of rIGFBP-2



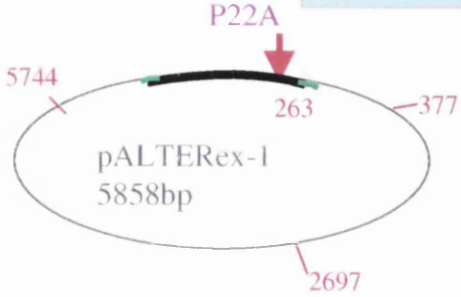


IGFBP-2 gene
980bps
35+15 bp from
pGEM polylinker

EXPECTED FRAGMENT SIZE

Sac-I

pALTER-IGFBP-2	pALTER-P20A
linearized	258> 928= 670
6838	6838-670=6168



EXPECTED FRAGMENT SIZE

PvuII

pALTER-IGFBP-2	pALTER-P22A
5745 >377 (+980) =1471	5744 >263 = 832
5744-2697 = 3047	263 +377 = 640
2697-377 = 2320	377 >2697=2320
	2697 >5744=3047

3.1.2 Expression of wt, P20A and P22A IGFBP-2 mutated proteins

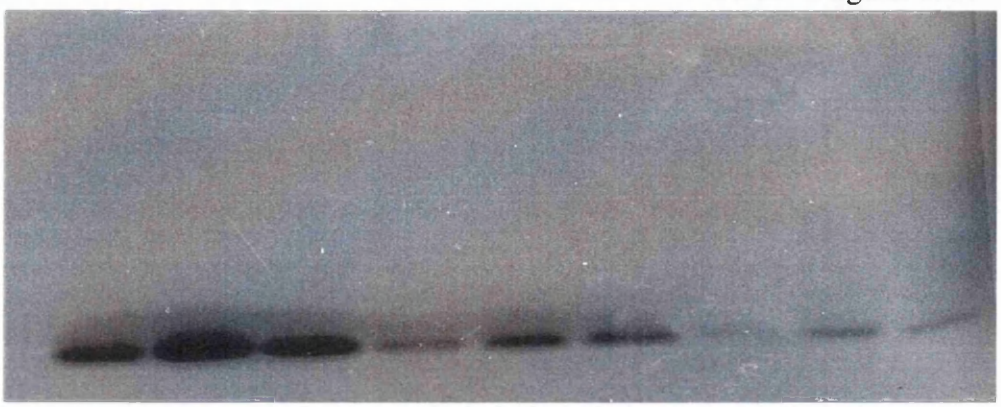
We chose to express wt IGFBP-2 and P20A and P22A mutants in insect cells using the baculovirus expression system. We initially considered expressing the protein in procaryotes (*E.coli*), as IGFBP-2 is not post translationally modified and bacterial expression of IGFBP-3 had been reported in the literature (Mohseni Zadeh and Binoux, 1997a,b). However, IGFBPs being highly disulphide bonded (9 S-S bridges), we expected that complicated purification, denaturation and refolding steps would be required in order to yield a functional protein. The Baculovirus expression system offered the advantage of being able to harvest the recombinant protein in a soluble form directly from the insect cell conditioned medium, as these cells are able to utilise mammalian signal peptide and secrete the protein.

Following the construction of recombinant baculoviruses carrying either wt IGFBP-2, P20A or P22A mutant DNAs, the expression of the encoded proteins was obtained by infecting Sf9 insect cells, as described in Mats and Meths. To detect the presence of wt IGFBP-2, P20A or P22A proteins in the insect cell supernatants, equal volumes of the concentrated conditioned media were electrophoresed on a 12% gel under non reducing conditions. Proteins were transferred to Hybond-C extra membranes, which were processed for ^{125} I IGF-I ligand blot, as described. The same membranes were subsequently reused for anti IGFBP-2 Western blot and protein bands were revealed by ECL technique. Wt IGFBP-2, P20A, P22A proteins were all detected by anti-IGFBP-2 antibody (Fig *3.1.2* **b**) and they appeared as a major band of approximately 36kDa. A lower intensity band of smaller Mr is detectable in the P20A preparation and it probably represents a proteolytic fragment of P20A. ^{125}I IGF-I ligand blots (Fig.*3.1.2.***a**) demonstrated that not only are insect cells able to secrete the binding proteins, but that probably wt IGFBP-2, P20A and P22A are also correctly folded as they retain IGF-binding ability. No smaller Mr bands are detected with ^{125}IGF-I ligand blot technique. In both Western and ligand blot, P20A was the most concentrated sample followed by P22A and wt IGFBP-2. This result gives a preliminary indication that the 2 mutations introduced in IGFBP-2 did not affect IGF-I binding. In fact, band density detected with the IGF-I ligand blot, which partly depends on the affinity of the binding proteins for IGF-I, substantially replicated the picture obtained with anti IGFBP-2 Western blot, which is not affected by the IGF binding characteristics of the proteins.

Fig *3.1.2*

Expression of wt IGFBP-2 , P20A and P22A mutants

^{125}I IGF-I ligand blot

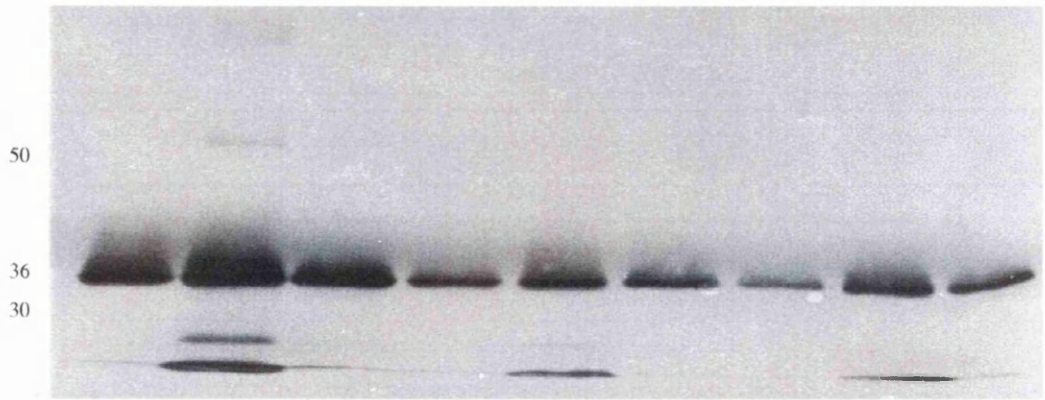


BP-2 P20A P22A BP-2 P20A P22A BP-2 P20A P22A

1 µl 0.5 µl 0.25 µl

a) Detection of protein expression in concentrated supernatants of insect cells cultures transfected with either IGFBP-2, P20A or P22A recombinant baculovirus. Equal volume of each sample was run in a 12% gel under non reducing conditions. Proteins were then transferred to nitrocellulose membranes and ^{125}I IGF-I ligand blot was carried out as described in Mats &Meths

Anti IGFBP-2 Western blot



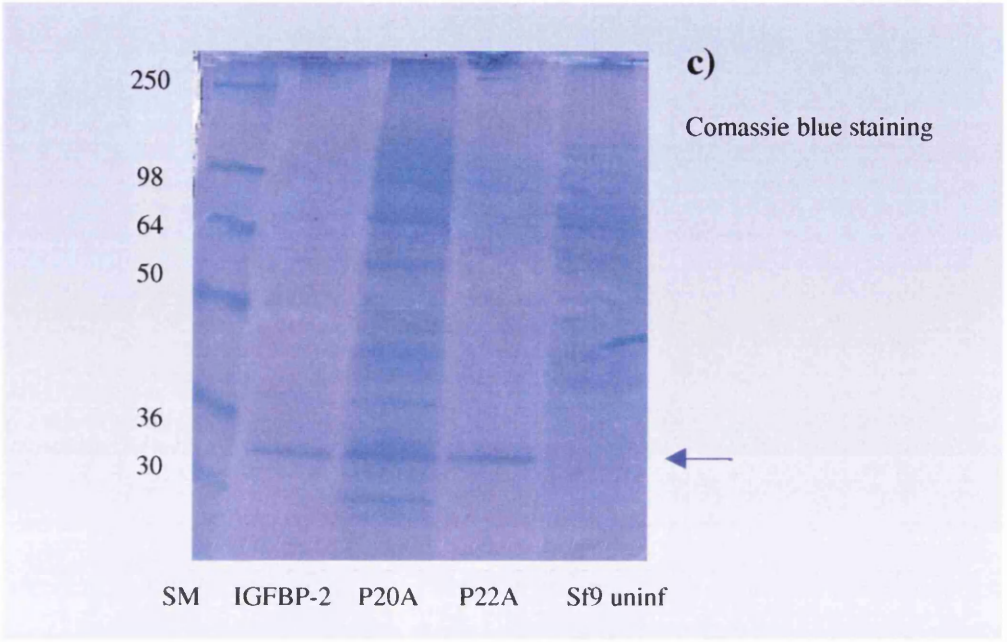
BP-2 P20A P22A BP-2 P20A P22A BP-2 P20A P22A

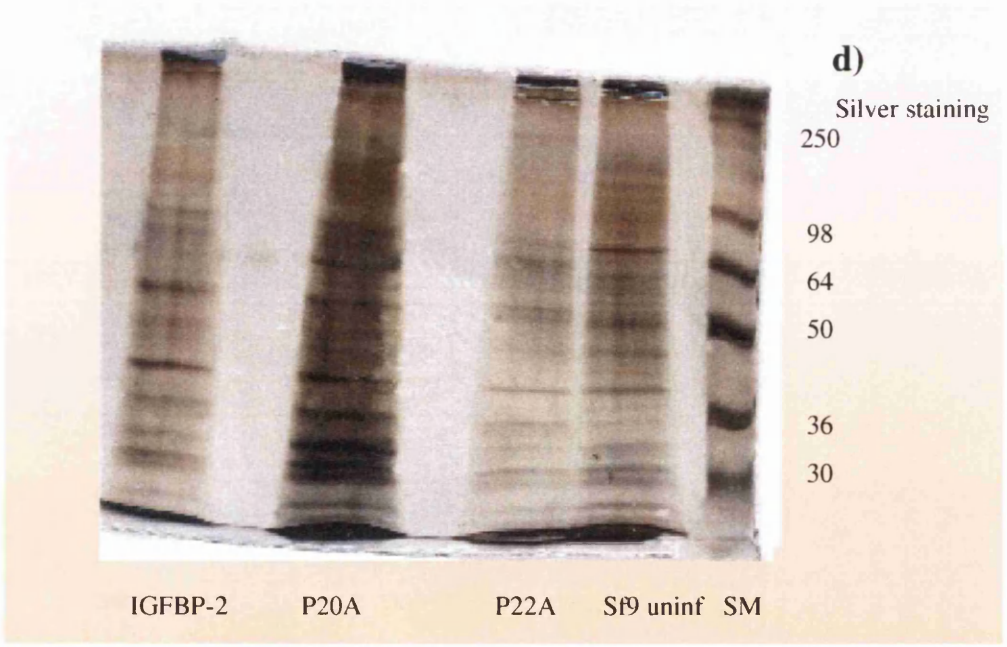
1 µl 0.5 µl 0.25 µl

b) This picture shows the same membrane blot utilized above subsequently processed for an anti IGFBP-2 Western blot.

Fig *3.1.2*

Expression of wt IGFBP-2, P20A and P22A





Detection of protein expression in concentrated supernatants of insect cell cultures transfected with either IGFBP-2, P20A or P22A recombinant baculovirus. A sample of concentrated supernatant of non-infected Sf9 cells (Sf9 uninf.) is also included as a control. Equal volumes of each sample were run in a 12% gel under non reducing conditions. The arrow in Fig.**c)** indicates the location of the putative IGFBPs

According to the manufacturers (Life Technologies), if insect cells are grown in serum-protein-free medium, recombinant secreted proteins can represent up to 95% of the total protein concentration of culture supernatants, provided that conditioned media are harvested before cell lysis occurs (Guide to baculovirus expression vector systems (BESV) and insect cell culture techniques-Instruction manual-GIBCO BRL). We assayed the level of purity of recombinant proteins in the concentrated insect cell supernatants by Coomassie and silver staining.

As shown in Fig *3.1.2.***c**, under our experimental conditions, Coomassie staining revealed that in each protein preparation the recombinant IGFBP protein was the predominant band. However it is difficult to estimate the relative concentration of the IGFBPs among all other contaminant bands. In silver staining (Fig *3.1.2.***d**), which is a more qualitative technique, a much higher number of protein bands was detected and it became difficult to identify clearly the band corresponding to the recombinant IGFBP.

3.1.3. IGF-affinity studies on the wt, P20A and P22A IGFBP-2 proteins

3.1.3.1. Competition studies with charcoal binding assay

As described in the Materials and Methods section (paragraph 2.1.3), in a preliminary study we constructed an ^{125}I IGF-I and ^{125}I IGF-II binding curve for wt IGFBP-2, P20A and P22A in order to analyse the relative binding capacity of equivalent amounts of recombinant protein preparations. On the basis of these data we were able to choose the appropriate volume of IGFBP preparations to be used in the subsequent competition study. Two criteria influenced this choice: i) the volume chosen for each IGFBP preparation should lie in the linear part of the IGF-binding curve ii) this amount of IGFBPs should bind a relatively low percentage of IGF tracer (approx. 20%). These conditions would guarantee that in the competition studies IGFBP would be saturated with ^{125} I IGF-I or II so that the addition of cold IGF competitor would displace tracer from the binding protein.

The competition studies aimed to determine the relative affinity of wt IGFBP-2, P20A and P22A for IGF-I or IGF-II. Fixed volumes of recombinant IGFBP-2, P20A or P22A preparations were incubated with a fixed amount of ^{125}I IGF-I/II tracer competed by increasing concentrations of cold IGF-I or IGF-II (0.25, 0.5, 1, 2.5, 5, 10, 25, 50ng/ml). Different ranges of cold IGF competitors were tested in previuos

experiments which showed similar results with respect of the affinity of IGFBP-2 and its 2 mutants for IGFs. Data presented here show the results of a single experiment which was chosen as representative example. The competition curves obtained are shown in Fig. *3.1.3.1***a**, **b**, **c** and **d**. Data were plotted as follows: on the X axis the increasing concentration of cold IGF (ng/ml) is plotted: on the Y axis is reported the amount of bound tracer expressed as % of cpm controls (control= tracer+IGFBP in the absence of cold IGF, as described in Material and Methods). From these graphs, the IC_{50} values (concentration of cold IGF-I or II able to displace 50% of the tracer) were graphically derived. For all proteins, IC_{50} values fell in a range of 4-6ng/ml, except for wt IGFBP-2 where slightly lower (IC_{50}=2.5ng/ml) values were found when cold IGF-II was used as competitor of either IGF-I or IGF-II radioligand. Although this observation is not statistically significant, it is in agreement with the work of Oh *et al.* (1993a) who previously reported a 2 fold higher affinity of hIGFBP-2 for IGF-II in comparison to IGF-I.

Data were analysed statistically with GENSTAT 5 version 4.1.

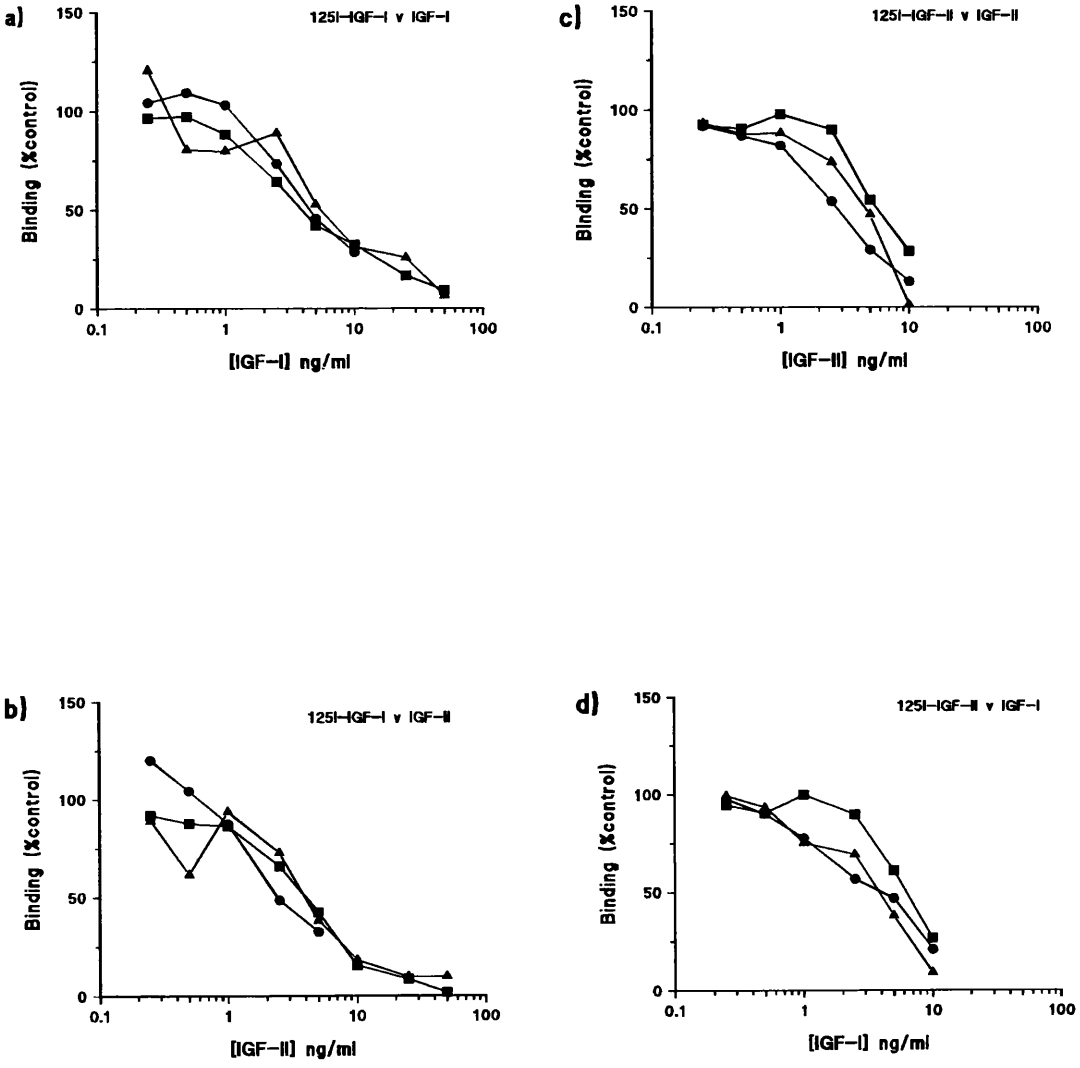
First, all competition curves (Fig. *3.1.3.1***a**, **b**, **c** and **d**) were smoothed by fitting into g-logistic curves and unnormalised analysed as A620 versus the log of the concentration, using *non linear regression analysis*. The IC_{50} values were calculated based on these fitted curves and they all ranged between 2.4 and 6.5 ng/ml (and again the lowest IC_{50} values (2.4 and 2.9ng/ml) were displayed by wt IGFBP-2 when cold IGF-II was used to compete either IGF-I or IGF-II tracer). A general analysis of variance (up to the second level of interactions) was applied to the IC_{50} data. None of the factors (protein, cold, and radiolabelled tracer), or the two level interactions between them, was significant.

Data were also transformed according to Scatchard's equation (Scatchard, 1949) and plotted as follows: the amount of bound IGF-I or II (B) was expressed in pM on the X axis, while on the Y axis the ratio between bound and free IGF-I or II (B/F) was plotted. A *linear regression analysis with groups* was applied to the transformed data and the gradient of the slopes (representing the Kd value of IGFBPs for IGF-I or II) shown to be not significantly different.

In conclusion, our analysis show no evidence that single mutations of the conserved prolines located at position 20 or 22 of rat IGFBP-2 affect IGFBP-2 binding to IGF-I or II.

Fig *3.1.3.1*

Affinity studies of wt IGFBP-2, P20A and P22A for IGF-I or IGF-II



Charcoal binding assay. Radio-labelled IGF-I or-II (25000cpm/tube) was incubated with a fixed amount of the insect cell supernatant containing either • wtIGFBP-2, ■ P20A or ▲ P22A. The tracer was competed with increasing amount of cold IGF-I or –II, as indicated on the x axis. Data on the y axis express tracer binding as % of control samples (IGFBP+ tracer in the absence of cold competitor). Each point shown in the graphs represents the mean of three replicates.

3.1.3.2. DISCUSSION

While the IGFBP binding site on the IGF molecule has been extensively studied and fairly well characterized (Bayne *et al.*, 1988; Baxter *et al.*, 1992; Heding *et al.*, 1996) the identification of IGF binding sites on IGFBP is still the object of study and debate. Site-directed mutagenesis, deletion of protein domains and the utilisation of naturally occurring proteolytic fragments were some of the strategies employed to identify that the IGF binding site is either in the N-terminus or C-terminus of IGFBP molecules. Contradictory results for the involvement of one or other domain were reported by different groups of researchers (Brikman *et al.*, 1991a; Lalou *et al* 1996; Hashimoto *et al.* 1997; Firth *et al.*, 1998; Ho and Baxter 1997a; Forbes et al., 1998). It is now generally believed that although either one or the other domain of IGFBPs might play a pivotal role in IGF binding, both the N- and C-terminus of IGFBPs are necessary to achieve the typical high affinity binding to IGFs (Kalus *et al.*, 1998; Qin *et al.*, 1998). The occurrence of binding in multiple steps or the existence of distinct binding sites for IGF-I or IGF-II has also been proposed (Roghani *et al.*, 1991; Ho and Baxter, 1997a; Forbes *et al.*, 1998).

As far as IGFBP-2 is concerned, studies aiming to identify the IGF binding site have been published only very recently and its location in either the N- or C-terminal domain has been supported. Hobba et al. (1996 and 1998) showed that iodination of a tyrosine residue at position 60 or its substitution with an alanine caused a 4 and 8 fold decrease in the affinity for IGF-II and IGF-I respectively. On the other hand Ho and Baxter (1997a) showed that a 14kDa C-terminal fragment of IGFBP-2 retained IGF binding capacity, while the deletion of the most C -terminal 62 residues severely compromised IGF binding (Forbes *et al.*, 1998).

At the time we started our research much of this information was not available, neither was it known if the N-terminus and the C-terminus of IGFBPs represented two distinct domains or if they were cross-linked through disulphide bonds forming a functionally indivisible domain. Observing the aligned sequences of different IGFBPs (Fig *3.1.3.2*), we noticed that at position 20, 21 and 22 of mature IGFBP-2 there was a group of three prolines, well conserved among IGFBP species. We hypothesised that these proline residues might have been preserved from changes throughout evolution because they played a functional role and perhaps had a part in IGF-binding, the best known of the IGFBP functions. Therefore we decided to investigate if single mutations of proline residues at position 20 or 22 of the wt IGFBP-2

Fig *3.1.3.2* Alignment of IGFBP sequences in their N terminal domains

protein affected its affinity for IGF-I or -II. Our results indicate that the substitution of these prolines with alanine residues has no effect on IGF-I or IGF-II binding.

Other data from our competition studies indicate the relative affinity of wt IGFBP-2 for IGF-I or IGF-II. Although when calculated from the raw data, the IC_{50} value of IGFBP-2 for IGF-I (in the presence of either ^{125}I IGF-I or ^{125}I IGF-II) was double that of the IC_{50} value for IGF-II (in the presence of either the radio-ligands), the statistical analysis of the results of the competition binding studies, revealed no significant difference in affinity of any of the wt or mutant IGFBP-2 proteins for either of the growth factors. A higher affinity of IGFBP-2 for IGF-II in comparison to IGF-I has been reported in the literature. IGFBP-2 preference for IGF-II was found to be as high as 20-30 fold or as little as 2-4 fold (Rosenfeld *et al.* 1989; Roghani *et al.*, 1991; Oh *et al.*, 1993a). Discrepancies could be due to different experimental conditions employed. When biological fluids are used as a source of IGFBP-2, it is possible that the presence of contaminant IGFBPs with very high affinity for IGF-II might have influenced the results. Vice versa, the method utilised for IGFBP-2 purification may have caused loss of its preference for IGF-II. Our data fall in the same order of magnitude of those obtained by Oh *et al.* (1993a) who found that hIGFBP-2 IC_{50} values for IGF-I were respectively 5.5 and 9.5ng/ml when ^{125} I IGF-I or II were used, while the IC_{50} values for IGF-II were respectively 5.0 and 6.5ng/ml when ^{125} I IGF-I or II were used.

During the course of our studies, research in this area made remarkable progress and new lines of investigation were opened as it became clear that in a biological context the interaction between IGFs and their binding proteins was not simply a matter of affinity, but was in fact the result of an intricate equilibrium between multiple factors. For instance specific IGFBP proteases present in biological liquids or the association of IGFBPs with the cell surface/ECM were shown to significantly affect IGFBP affinity for IGFs. Moreover it was found that IGFBPs could exert not only an inhibitory but also an enhancing modulation of IGF action or even an IGF-independent effect. Therefore we decided to continue our studies on IGF-IGFBP-2 interactions in a cell culture model which would offer a more biological context.

3.2 IGF-II affinity chromatography purification of wt IGFBP-2 and PGD IGFBP-2 mutant

With a view to future studies aiming to investigate the biological functions of IGFBP-2 in a cell culture system, we proceeded to purify wt IGFBP-2 from the concentrated insect cell supernatant.

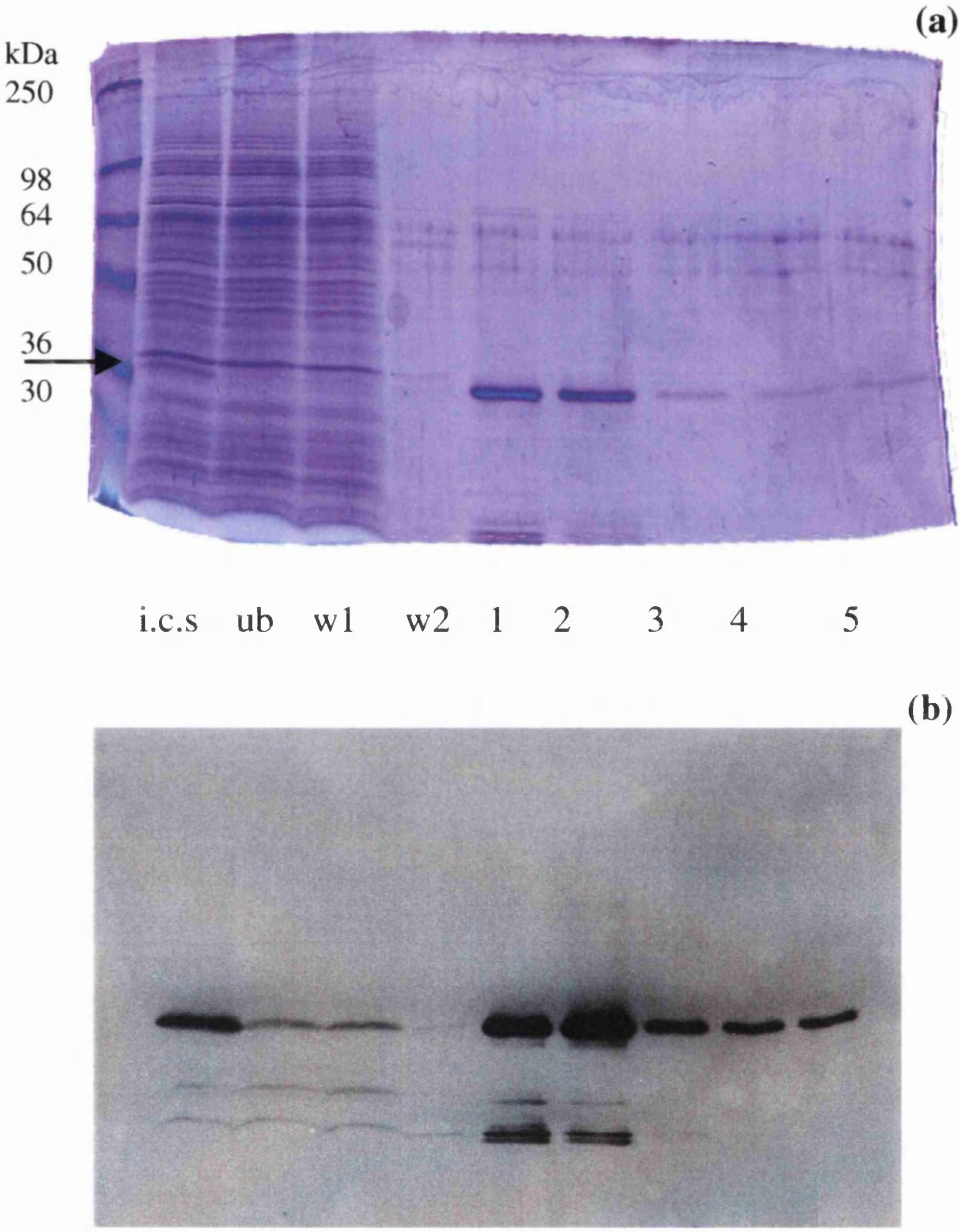
Fig *3.2* **a** and **b** show the purification of IGFBP-2 following the processing of concentrated insect cell supernatant on an IGF-II affinity chromatography column. Samples from each purification step were electrophoresed on a 12% gel and protein bands were detected by Coomassie blue staining (Fig *3.2.***a**). Most of the contaminant proteins present in the starting material were recovered in the unbound and washing fractions, while fractions eluted with 0.5M acetic acid pH 3, principally contained a 36kDa protein and a lower Mr species. The identity of the eluted protein and a proteolytic fragment from this protein was confirmed with an anti IGFBP-2 Western blot (Fig *3.2.***b**) which also showed the enrichment in IGFBP-2 concentration in the first elution fractions compared with the starting material.

In addition, we also expressed and purified the PGD-IGFBP-2 mutant, which was generated through site directed mutation of the wt IGFBP-2 cDNA by Dr G. Allan in our laboratory. The C-terminal RGD integrin-binding sequence was mutated to PGD in order to investigate the possible involvement of the RGD sequence in IGFBP-2 association with the cell surface. Similarly to the corresponding wt protein, PGD-IGFBP-2 was purified on an IGF-II affinity column. Purification steps, as analysed by Coomassie blue staining and anti IGFBP-2 Western blot, are shown in Fig *3.2.***c** and *3.2.***d** respectively.

As an example of the degree of purification achieved with IGF-II affinity chromatography, a silver stained gel of the purification steps of P20A IGFBP-2 is also shown in Fig *3.2.***e.**

Fig 3.2

IGF-II affinity chromatography purification of rat IGFBP-2

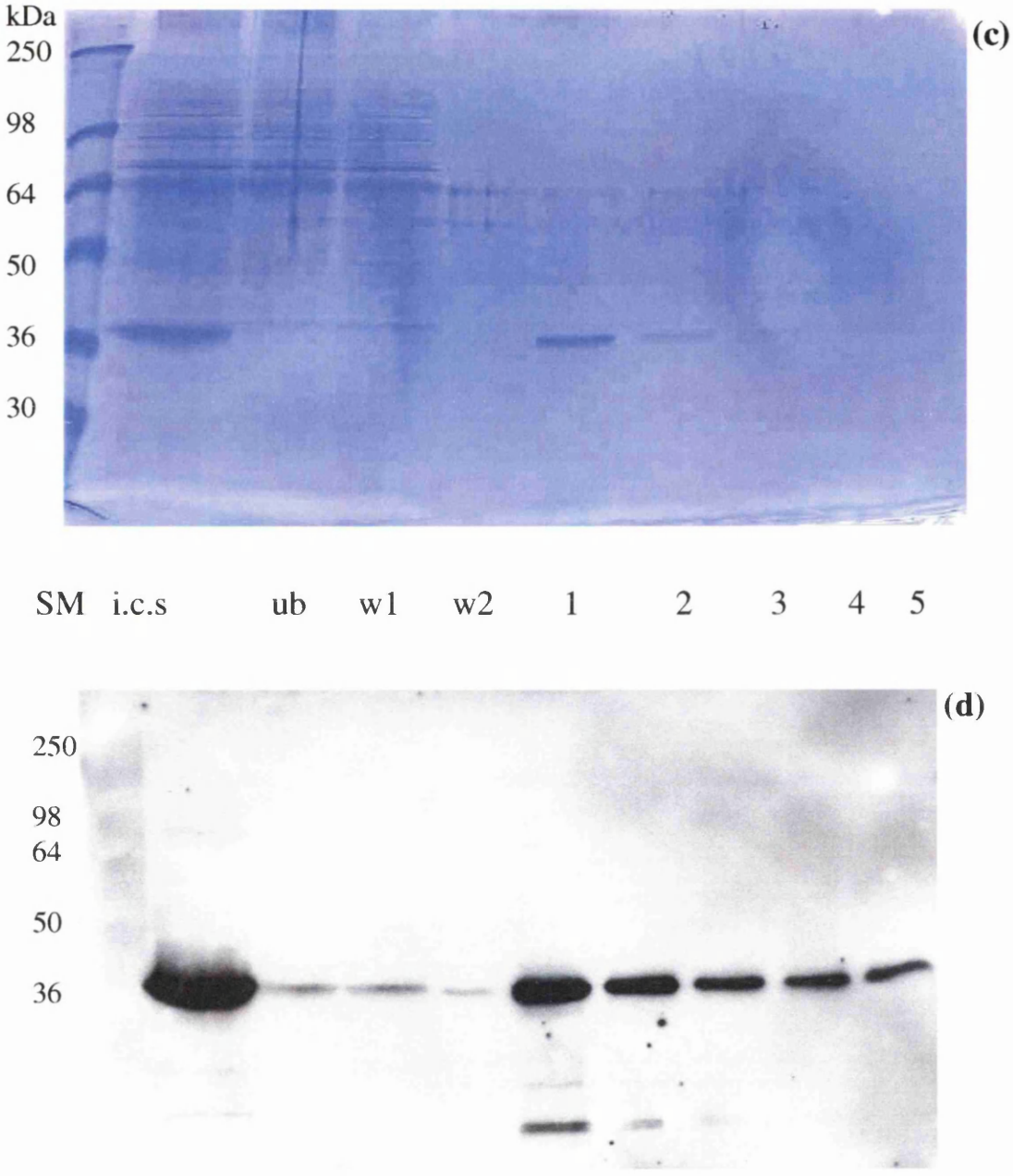


IGF-II affinity chromatography purification steps of IGFBP-2.

Concentrated insect cell culture supernatant (I.c.s) was loaded onto the the IGF-II affinity column, the unbound material (ub) was collected and the column was washed twice with the equilibrating buffer. Elution was performed with 0.5M acetic acid and the first 5 fractions were analysed for the presence of IGFBP-2. Samples were taken from each purification step and electrophoresed on a 12% gel under non denaturing conditions.Comassie blue gel staining is shown in the top picture. An anti IGFBP-2 Western blot is shown in the bottom picture.

Fig 3.2.

IGF-II affinity chromatography purification of PGD-IGFBP-2

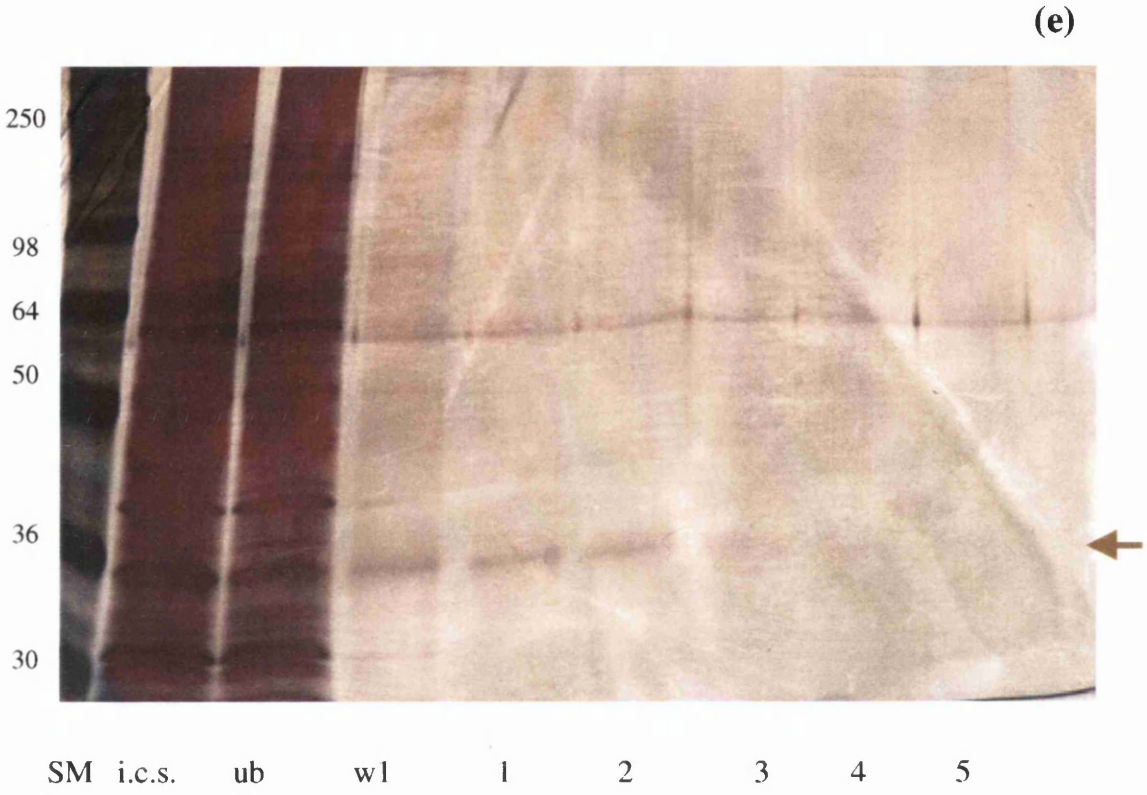


IGF-II affinity chromatography purification of PGD-IGFBP-2.

Concentrated insect cell culture supernatant (I.c.s) was loaded onto the the IGF-II affinity column, the unbound material (ub) was collected and the column was washed twice with the equilibrating buffer. Elution was performed with 0.5M acetic acid and the first 5 fractions were analysed for the presence of PGD-IGFBP-2. Samples were taken from each purification step and electrophoresed on a 12% gel under non denaturing conditions.Comassie blue gel staining is shown in the top picture. An anti IGFBP-2 Western blot is shown in the bottom picture.

Fig *3.2*

P20A affinity chromatography purification



P20A-IGFBP-2 purification steps on an IGF-II affinity chromatography column.

Concentrated insect cell culture supernatant (i.c.s) was loaded onto the IGF-II affinity column, the unbound material (ub) was collected and the column was washed twice with equilibrating buffer (the first ml run through the column was collected, w1). Elution was performed with 0.5M acetic acid and the first 5 fractions (1-5) were harvested. Samples were taken from each purification step and electrophoresed on a 12% gel under non denaturing conditions.
Protein bands were then detected by silver staining.
The arrow indicates the location of the putative P20A-IGFBP-2

3.3.Studies on biological features of IGFBP-2

We focused our research on the investigation of possible IGFBP-2 functions other than simple inhibition of IGF action. As already mentioned in the introduction chapter, it is believed that IGFBP association to the cell surface could be important for the IGF-potentiating or IGF-independent effects described for some IGFBPs (De Mellow and Baxter, 1988, Conover, 1992). Although IGFBP-2 principally shows an IGF-inhibiting effect (Clemmons, 1997), it has been hypothesised that cell membrane-associated IGFBP-2 could in fact facilitate IGF binding to IGF-IR (Guenette and Tenniswood, 1994). We aimed to establish an experimental model which would allow us to investigate the biological role of cell-associated IGFBP-2.

Although IGFBP-2 is produced by many tissues and cell lines, at the time as this study was undertaken, its interaction with the cell surface had been described in only a very few cell types.

- In *section 3.3.1* we tried to identify a cell line on which IGFBP-2 binding with the cell surface could be demonstrated. Two opposite approaches were followed in these studies: the addition of exogenous recombinant rat IGFBP-2 to cultured cells (*section 3.3.1.a.*), or the detection of endogenous IGFBP-2 naturally associated with cell surface (*section 3.3.1.b*). Membrane-bound IGFBP-2 was demonstrated in Clone 9 cells.
- In *section 3.3.2* we then focused on finding a system to reveal Clone 9 cell responsiveness to IGF and, at the same time, the influence that soluble or monolayer associated IGFBP-2 exerts on IGF action
- In *section 3.3.3.* we constructed a model suitable

  -to study biological functions of IGFBP-2 in Clone 9 cells under basal conditions

  -or to investigate the specific effect of cell membrane-associated IGFBP-2 on IGF stimulation.

3.3.1.Studies on IGFBP-2 association to the cell surface

3.3.1.a Screening different cell types and different methods to detect the association of exogenously added recombinant rat IGFBP-2 with the cell surface.

In this part of the project we tried to determine if exogenous recombinant IGFBP-2 could associate with the cell surface. Intuitively this would appear the most direct way to demonstrate such binding and many studies published on IGFBP-3 and -5 association with the cell surface (Smith *et al.*; 1994; Karas *et al.*, 1997; Yang *et al.*, 1996 Abrass *et al.* 1997; Andress, 1998) or to ECM (Parker *et al.*, 1996; Arai *et al.*, 1996) utilised this approach. To our knowledge no published reports demonstrated evidence of exogenous IGFBP-2 binding to membranes of cultured cells, but we had no reason to believe this technique would be unsuitable for this purpose. Moreover, if radioactive IGFBP-2 was used as a probe, the method could achieve the sensitivity necessary to detect the very small amount of binding protein that we expected to be associated with the cell surface. On the other hand, a possible drawback of this method could be that in cell lines naturally producing IGFBP-2, cell surface binding sites could be already saturated by the endogenous protein.

Unexpectedly, we found many difficulties associated with the use of recombinant IGFBP-2 and we failed to obtain convincing proof of exogenous IGFBP-2 binding to cell surfaces. Therefore, here (table *3.3.1.a.1-5*) we present only a brief description of the rationale and results of the experiments which represent to a large extent attempts to overcome technical problems. A detailed description of every single experiment can be found in the Materials and Methods chapter, while an overall discussion of the results is presented at the end of this section.

Table *3.3.1.a.1-5*

Cell type	*Rationale and Results*

EXPERIMENT *3.3.1.a.1 Use of an immunohistochemical technique (anti IGFBP-2 antibody) to detect recombinant rIGFBP-2 or PGD-IGFBP-2 mutant binding to Dx3 cell monolayer*

Dx3, human skin melanoma cells, express $\alpha_{II}\beta_3$ and $\alpha_v\beta_3$ integrins on the cell membrane	This experiment was undertaken as part of studies on IGFBP-2 interaction with integrins (Exp. 3.3.1.a.9). Dx3 cells were chosen as, once IGFBP-2 binding to the cell surface had been demonstrated, it would have been possible to further investigate if $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins were involved in IGFBP-2 binding. Use of an immune detection technique, could potentially be exploited to detect membrane association of both wt and PGD IGFBP-2. *Results:* -The addition of recombinant IGFBP-2 or its mutant PGD IGFBP-2 led to a background binding which was much higher in cell-free compared to cell-coated wells. Therefore wt or PGD IGFBP-2-binding detected with this method seems to be due to a direct association of the binding proteins to tissue culture plastic.

EXPERIMENT *3.3.1.a.2.* ^{125} *I IGFBP-2 binding to A10 and Clone 9 cell surface in competition with cold IGFBP-2*

A10, rat smooth muscle cells Clone 9, adult rat liver cells Both cell lines naturally secrete IGFBP-2	IGFBP-2 association with the cell surface had been described in the literature to a limited extent (Reeve *et al.*, 1993, Russo *et al.*, 1997). In both cases, IGFBP-2 was endogenously produced by the cells. We thought that an IGFBP-2 secreting cell line may be more likely to express also the appropriate binding sites on the cell surface *Results:* -The background of IGFBP-2 tracer bound to plastic was higher than the amount of tracer bound to cells. -Cold IGFBP-2 did not displace the tracer. -To test the method, we also tried to detect ^{125}I IGF-I tracer binding to cell membranes (IGF receptors (including possible IGFBPs associated to the membranes). In this case i) cold IGF-I effectively competed the tracer binding and ii) the back ground radioactivity was slightly lower in the absence than in the presence of the cells.

EXPERIMENT *3.3.1.a.3 Stripping endogenous IGFBP-2 potentially bound to the cell membrane before incubating the cells with* ^{125}*I IGFBP-2*

A10 Clone 9	The inability to detect any binding of IGFBP-2 tracer to the cell surface could be due to the saturation of the binding sites by endogenous IGFBP-2 (especially in Clone 9 cells where IGFBP-2 secretion is high). *Results* -Despite stripping treatment, tracer bound in the presence of cells did not exceed tracer background bound to plastic. -Cold IGFBP-2 was unable to displace the tracer

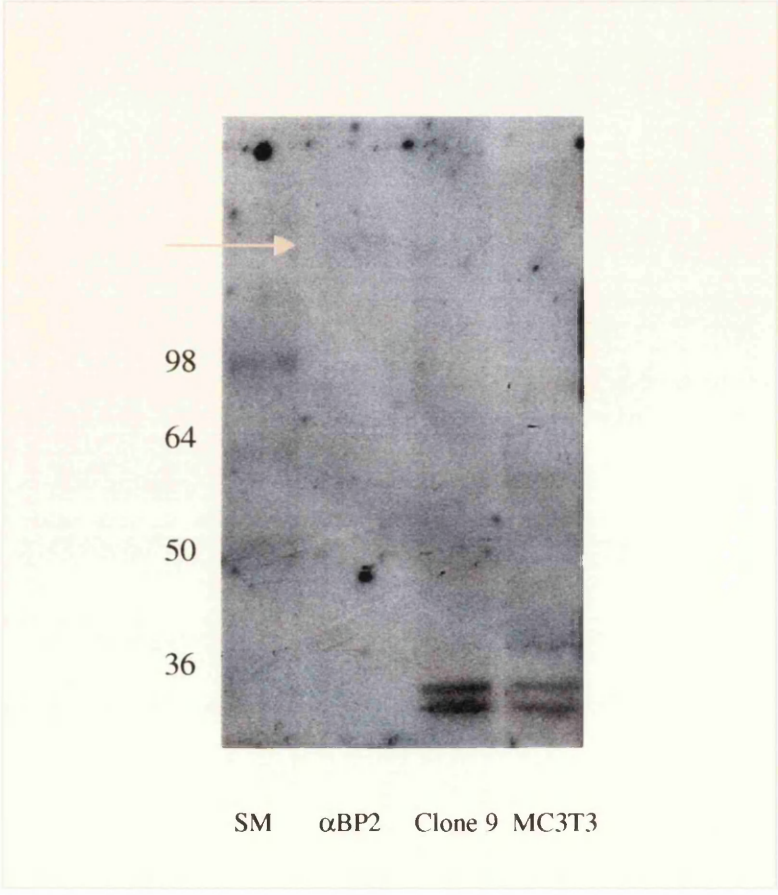
Cell type	*Rationale and Results*
EXPERIMENT *3.3.1.a.4.* ^{125} *I IGFBP-2 binding to cell monolayers cultured on positively charged plates*	
Clone 9	It has been described that positively charged plates reduce the background binding of IGFBP-5 to plastic surface to <3%, compared to 15% for negatively charged plates (Jones *et al*, 1993). We tested if these plates were suitable to reduce also ^{125}I IGFBP-2 binding background. *Results* -background of IGFBP-2 tracer bound to positively charged plastic was still higher than the tracer bound to cell monolayer -Cold IGFBP-2 was unable to displace tracer.
EXPERIMENT *3.3.1.a.5* ^{125} *I IGFBP-2 binding to cells in suspension*	
A10 3T3F442A, mouse preadipocytes. These cells were chosen because they do not secrete IGFBP-2 into the conditioned medium, therefore cell surface binding sites may be available for tracer binding	To reduce the background tracer binding to plastic plates and increase the cell surface available, cells, scraped from dishes and resuspended at high density in tubes, were incubated with ^{125} I IGFBP-2 tracer in the presence or absence of cold IGFBP-2 *Results* -background of IGFBP-2 residue in the no-cell tubes was higher than in cell pellets containing tubes. -cold IGFBP-2 was unable to displace the tracer from the cell pellet.

3.3.1.a.6 ^{125} *I IGFBP-2 ligand blots on Clone 9 and MC3T3-E1 cell membrane preps.*

Enriched plasma membrane preparations, obtained from Clone 9 or MC3T3-E1 cells as described in Materials and Methods, were electrophoresed on a 10% gel and blotted. After transfer, membranes were probed with radio-labelled IGFBP-2 and exposed to direct autoradiography (Fig *3.3.1.a.***6**). To test if the method was suitable to detect proteins known to be IGFBP-2 binders, an anti-IGFBP-2 antibody was included as a positive control. No obvious band appeared at 150kDa, although in a previous 7.5% gel (data not shown) the anti IGFBP-2 antibody was revealed as one major 150kDa band and 2 fainter bands with higher Mr. Conversely, in both Clone 9 and MC3T3-E1 ECM tracks, the IGFBP-2 tracer clearly detected a doublet of approximately 34-36kDa. The different intensity of the bands in the two cell types could be due to different protein concentration in the membrane preps or to a difference between cell types. It is of

Fig *3.3.1*.a.6

^{125} I IGFBP-2 ligand blot of Clone 9 and MC3T3-E1 plasma membranes



^{125}I IGFBP-2 ligand blot of Clone9 and MC3T3-E1 plasma membranes.

Membranes were prepared as described in Mats & Meths, electrophoresed in a 10% gel and blotted onto Hybond C-extra membranes. Blots were further processed for ^{125}I IGFBP-2 ligand blot and finally exposed to an autoradiograph film for 10 days. An anti IGFBP-2 antibody was included as a control of the method. The arrow indicates the expected position of the anti IGFBP-2 band (αBP-2).

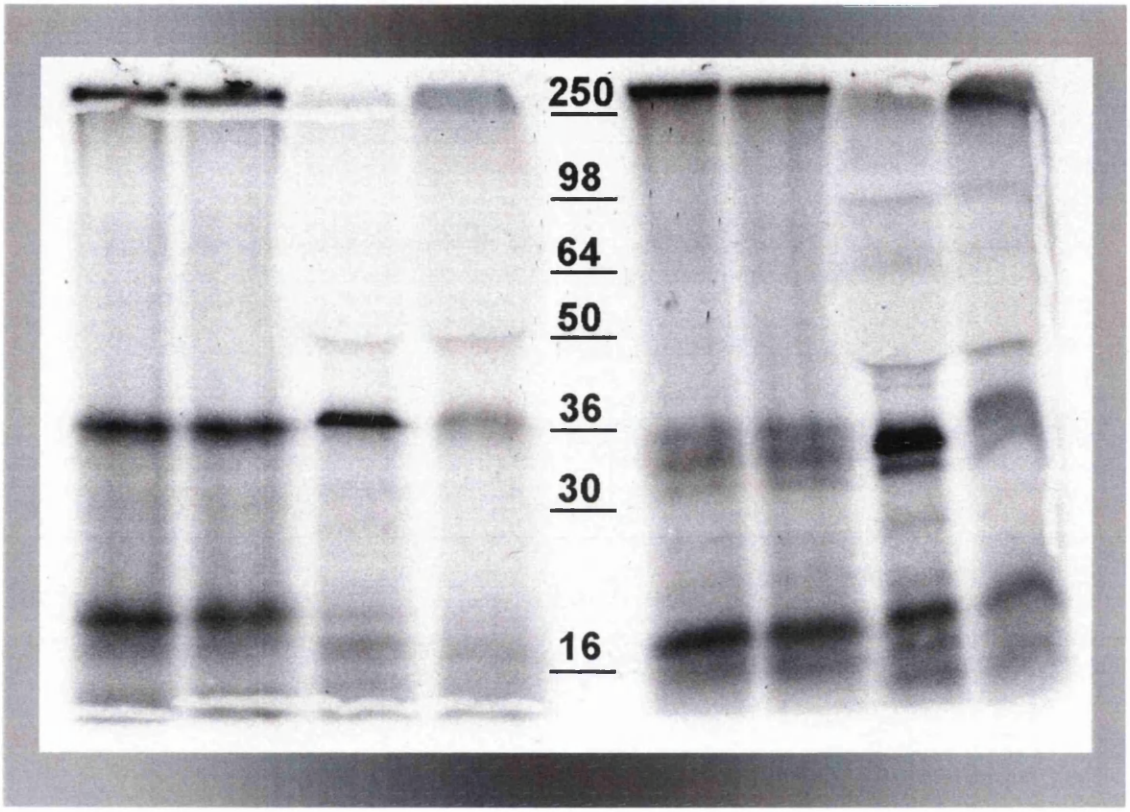
interest that, among several other bands, a 34-36kDa doublet was also visible with Ponceau S staining. If the double band displayed by IGFBP-2 ligand and by Ponceau S staining represented the same protein/s, the abundance of this protein would be high. Furthermore, in the other half of the same blot, 1μg/ml of cold IGFBP-2 was included during incubation with ^{125}I IGFBP-2, but no competition was observed (data not shown) suggesting that perhaps the binding of the tracer to these 2 bands was not specific.
In conclusion the nature of the 34-36kDa doublet protein detected by ^{125} I IGFBP-2 ligand blot on Clone 9 and MC3T3-E1 membranes, remains unknown.

3.3.1.a.7 ^{125} I IGFBP-2 cross-linking to Clone 9 membranes treated +/- sodium chlorate.

As Russo et al. (1997) showed that IGFBP-2 bound to rat olfactory bulb plasma membranes through the mediation of glycosaminoglycans (GAGs), we tried to see if similar evidence could be obtained in Clone 9 cells. We prepared plasma membranes from Clone 9 cells cultured in routine conditions or in the presence of 30mM $NaClO_4$. This reagent is known to inhibit membrane GAG sulphation which in some cases results in a reduction of GAG binding capacity (Hoogewerf *et al.*, 1991). In order to test if $NaClO_4$-treated plasma membranes showed a reduction in ^{125}I IGFBP-2 binding, both normal and treated plasma membranes were incubated with the tracer. In addition, as it was also conceivable that membrane association of ^{125}I IGFBP-2 was mediated by membrane proteins, we added DSS, an homobifunctional cross-linking agent, to stabilize this potential binding. Cross-linked samples were analysed by electrophoresis performed either under reducing or non reducing conditions. Samples of IGFBP-2 tracer alone self cross-linked in the presence or absence of heparin were also included. With these controls we intended to check if the cross-linking reaction led to the formation of i) IGFBP-2 dimers, whose occurrence during protein purification has been reported (Bourner *et al.*, 1992), or ii) putative aggregates between IGFBP-2 tracer and a GAGs, like heparin. The DSS cross-linking agent is active on primary amines, therefore it could stabilise IGFBP-2 dimers, if present, but it was expected to fail to cross-link heparin and IGFBP-2. As shown in Fig *3.3.1.a.*7 under non reducing conditions ^{125}I IGFBP-2 tracer migrated as a single band of approximately 36kDa and no dimer forms were detected. Under reducing conditions ^{125}I IGFBP-2 migrated with an apparent Mr of 34kDa, and two additional bands were apparent. One, just below the main 34kDa band, was present in all samples run under reducing conditions. The other, an approximately 19kDa band, appeared in all tracks with the exception of the tracer under non

Fig *3.3.1*.a.7

^{125} I IGFBP-2 cross-linking to Clone 9 plasma membranes



Membrane preps from Clone 9 cells cultured in the presence or in the absence of 30mM sodium chlorate, were cross-linked with ^{125} I IGFBP-2. As control, the cross-linking reaction was done with IGFBP-2 tracer alone or in the presence of heparin. Samples were then electrophoresed on a 12% gel under reducing or non reducing conditions and dried gels were exposed to direct autoradiography.

reducing conditions. The addition of heparin to tracer sample during the cross-linking reaction, did not change the pattern of IGFBP-2 tracer bands either under non reducing or non reducing conditions but it decreased their intensity. The lower intensity may be explained by the appearance of a large Mr aggregate at the top of the gel in which the tracer was partially sequestered.

In the tracks of Clone 9 membranes, irrespective of the treatment of cells with 30mM sodium chlorate, the pattern of the bands was virtually identical to tracer control lanes. This suggests that the bands shown were just a residue of the added tracer which was not cross-linked, but bound or just physically trapped in the membrane pellets or associated to the tube walls and resolubilised in the sample buffer. The most evident difference between membrane and tracer electrophoretic patterns was the lack of the 19kDa band in IGFBP-2 tracer (with or without heparin) run under non reducing conditions. The only other difference between tracer and membrane lanes was the presence, at the top the gel of all membrane tracks in both reducing and non reducing conditions, of a large Mr aggregates. We conclude that IGFBP-2 cannot be cross-linked to Clone 9 membranes by using DSS.

3.3.1.a.8 Preliminary study to detect exogenous/endogenous IGFBP-2 binding to Clone 9 cell extracellular matrix

In the first part of this experiment we simply compared ^{125}I IGFBP-2 binding to ECM coated or empty wells. Positively charged plates were utilised for this experiment and Clone 9 and MC3T3-E1 ECM coated wells were prepared from cell monolayers as described in the Materials and Methods section. After a 48 hour incubation with ^{125}I IGFBP-2 and following extensive washing of the plates, residual radioactivity was extracted either with 1N NaOH or with 2% SDS and determined by γ counting (data not shown). No differences in ^{125}I IGFBP-2 binding to the two ECM types, nor between the reagents used to remove the radioactivity, were observed. Despite the use of positively charged plates, the background radioactivity associated with the plastic was higher than the radioactivity associated with the ECM, therefore it was impossible to draw any information on IGFBP-2 binding to Clone 9 and MC3T3-E1 ECM.

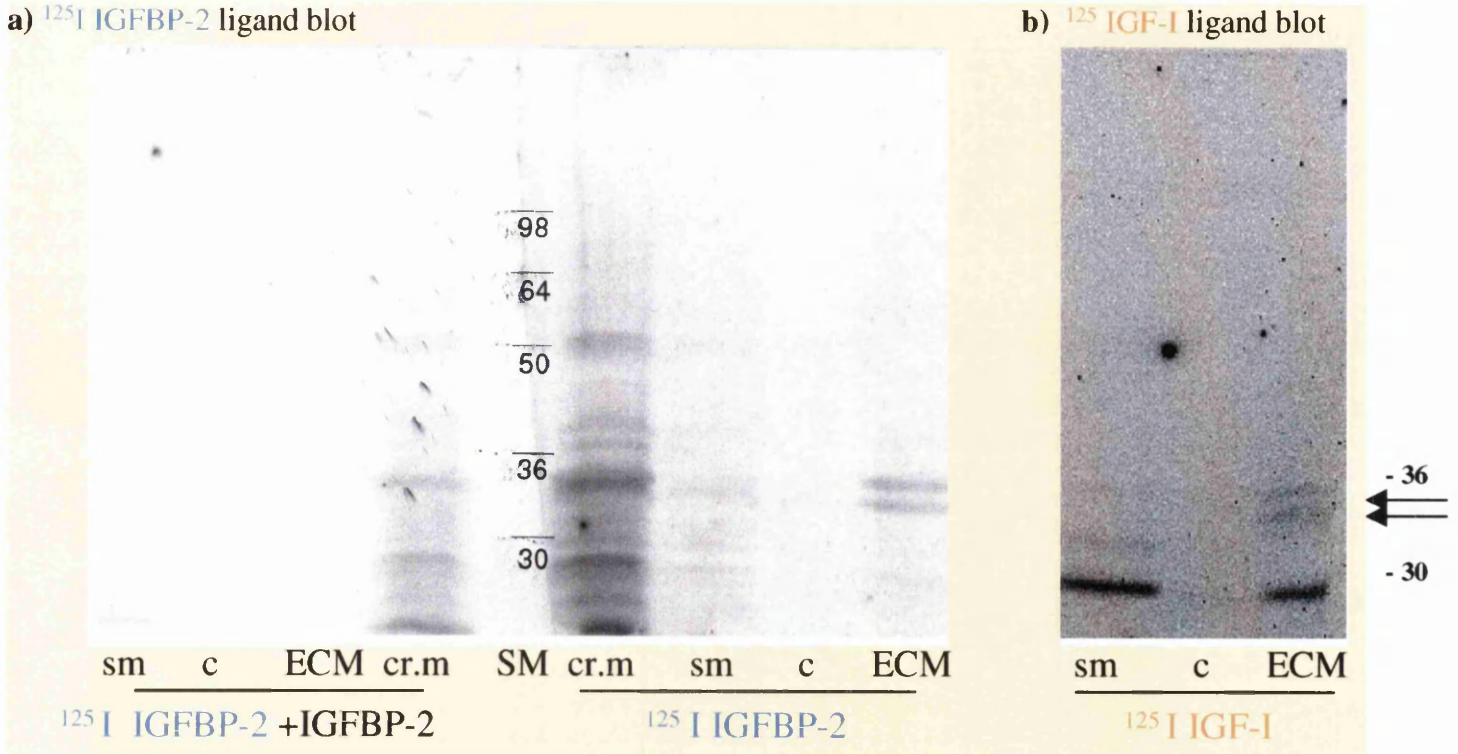
In the second part of the experiment, we investigated if any endogenous IGFBPs were present in Clone 9 ECM and, if this was the case, what kind of ECM molecule IGFBPs were bound to. Clone 9 cell

monolayers were sequentially treated with 0.5% TritonX-100 to solubilize the cell membranes and with 25mM NH_4 acetate pH9.0 to eliminate nuclei and cytoskeleton. Finally, the extracellular matrix was extracted with non reducing Laemmli sample buffer. Samples from each step of ECM preparation and samples of concentrated Clone 9 membrane preparation were electrophoresed, blotted and further processed for ^{125}I IGFBP-2 or ^{125}I IGF-I ligand blots as shown in. Fig *3.3.1.a.8* **a** and **b** respectively.

With the ^{125}I IGF-I ligand blot we aimed to detect the presence of natural IGFBPs possibly present on Clone 9 ECM. This experiment is presented in this section because the same samples were also analysed by ^{125}I IGFBP-2 ligand blot in the attempt to detect the ECM molecule capable of binding the tracer. We considered that it would be informative to compare the results obtained by the 2 techniques side by side. As shown in Fig *3.3.1.a.8* **b** (right hand side blot), radiolabeled IGF-I in both 0.5% Triton-extracted membrane and ECM preparations bound to a low Mr band (<30kDa), possibly an IGFBP proteolytic fragment with residual IGF-binding ability. In the ECM track a fainter but distinct double band, that migrated at approximately 32-34kDa, appeared and a faint shadow suggests it was possibly present also in the plasma membrane track. The band size, which corresponds to IGFBP-2, and the fact that IGFBP-2 is the only binding protein detectable in Clone 9 conditioned medium (CM) by ligand blot, suggests that this double band might represent, at least in part, IGFBP-2. However, we cannot exclude that the double band detected by ^{125}I IGF-I tracer represents an IGF-binding protein species, distinct from IGFBP-2, which is undetectable in CM, but is associated with Clone 9 cell membranes or ECM.

After the demonstration in Clone 9 ECM of an IGFBP, which we believed to be IGFBP-2, we attempted to identify the ECM molecule capable of IGFBP-2 binding, by ^{125}I IGFBP-2 ligand blot. The blot was divided in two halves and incubated with IGFBP-2 tracer in the presence or in the absence of cold IGFBP-2. As shown in Fig *3.3.1.a.8*.**a**), the left half membrane gave an image identical to the right part, but fainter as tracer binding was specifically competed by cold IGFBP-2. Along with samples of ECM preparation steps, a control sample of concentrated Clone 9 plasma membranes was included. Concentrated and 0.5% Triton-extracted membrane samples showed a very similar pattern, confirming the efficiency of the treatment with the detergent. In these tracks ^{125}I IGFBP-2 detected a multiplicity of bands, amongst which three darker areas appeared at about 50kDa, 34kDa and 28kDa. No bands were

Fig. *3.3.1.a.8* Comparison between ^{125} I IGFBP-2 ligand blot and ^{125}I IGF-I ligand blot on membranes and ECM preparations from Clone 9 cells



Clone 9 cells were cultured on Primaria 6 well plates and subsequently treated with i) 0.5%TritonX- 100 to solubilise and eliminate the cell membranes (sm); ii) with 25mM Na acetate to eliminate the cytoskeleton (c), and finally with iii) 2% SDS in order to solubilise and detach the ECM fraction from the plates. Samples from each fraction were run in a 12% gel, along with crude preparation of Clone 9 cell membranes (cr. m) for comparison. Gels were then blotted and processed for **a)** ligand blotting where ^{125} I IGFBP-2 or **b)** ^{125} I IGF-I were used as probes.

detectable in NH_4acetate-extracted samples corresponding to Clone 9 nuclei and cytosketelons. In the ECM sample track a distinct double band of approximately 32-34kDa appeared.

Interpretation of the bands detected by ^{125} I IGFBP-2 ligand is not easy as the use of this technique to identify IGFBP-2 binding molecules within a complex protein mix, such as membrane or ECM preparations, is novel and does not give guarantees on detection specificity. As will be discussed later, it was surprising to observe that ^{125}I IGF-I and ^{125}I IGFBP-2 ligand blots gave virtually the same picture.

3.3.1.a.9 Integrin binding studies

Table ***3.3.1.a.9*** summarises preliminary studies undertaken to investigate the interaction between IGFBP-2 and integrins.

Cell type	*Description of the experiment*
EXPERIMENT *3.3.1.a.9 a Fibronectin binding assay on K562 cells using IGFBP-1 as competitor*	
K562, erythroleukemia cells, express $\alpha_5\beta_1$ integrin on the cell membrane	The ability of IGFBP-1 to inhibit K562 cell adhesion to fibronectin coated wells was tested
EXPERIMENT *3.3.1.a.9 b Cell adhesion test on IGFBP-2 and IGFBP-1 coated plates*	
Dx3, human skin melanoma cells, express $\alpha_{II}\beta_3$ and $\alpha_v\beta_3$ integrins on the cell membrane	The ability of Dx3 cells, which express a wider range of integrins on their surface, to adhere to IGFBP-1 or IGFBP-2 coated wells was tested

In order to establish if the fibronectin binding assay was a suitable method to investigate IGFBP-2 interaction with integrins, IGFBP-1, which is known to bind to $\alpha_5\beta_1$ integrin, was utilised in a preliminary study. The fibronectin binding assay is based on the ability of various molecules to inhibit cell adhesion on a fibronectin substrate by binding and occupying cell surface $\alpha_5\beta_1$ integrin (fibronectin receptor). Undifferentiated K562 human erythroleukemia cells were employed as they express $\alpha_5\beta_1$ integrin (Conrad *et al.*, 1994; Zheng *et al.*, 1994). In addition to IGFBP-1, an RGD containing peptide and an anti β_1 integrin subunit antibody were used as competitors of cell attachment, while an RGE containing peptide and a mouse isotype antibody were used as negative controls. Duplicate values and the pattern of the positive controls were not consistent (except for RGD peptide which clearly inhibited cell attachment)

(Data not shown). Therefore no conclusions could be drawn on the effect of IGFBP-1 on K562 cell adhesion to fibronectin coated plates under our experimental conditions.

These experiments were highly consumptive of reagent, as inhibition of cell adhesion requires microgram quantities of competing protein. Therefore a different approach was attempted in the next series of experiments

In this case Dx3, a cell line derived from a human melanoma, was chosen because it expresses a wider range of integrin receptors, including $\alpha_{II}\beta_1$ and $\alpha_v\beta_3$ (vitronectin receptor) integrins (Helfrich *et al.*, 1992; Townsend *et al.*, 1999) and therefore could be more suitable for the initial screening. The ability of the cells to adhere directly to IGFBP-2 or IGFBP-1-coated wells was tested. We found no differences in the number of cells attached on plastic alone or on IGFBP-1 or IGFBP-2 coated wells.

3.3.1.a.10 DISCUSSION

The common feature of the experiments presented in this section was the attempt to identify IGFBP-2 association to plasma membranes or ECM by the addition of exogenous recombinant protein. The experiments described above failed to provide a conclusive answer on this matter and a general discussion of all results will be presented

Negative results could be due to the inability of IGFBP-2 to bind to cell membrane/ECM in the cell lines tested, because the binding sites were either absent, or saturated by endogenous IGFBPs. Alternative explanations could be that IGFBP-2 binding to cell surface/ECM could have occurred if different experimental conditions (incubation buffers, time and temperature) had been employed, or that it did occur, but the analytical methods we adopted failed to detect it. As no reports were available in the literature, we tested IGFBP-2 association to the cell surface by using the same conditions described for IGFBP-3 and IGFBP-5. It should be pointed out that even in the case of IGFBP-3, whose ability to bind to the cell surface is well documented in the literature, only 6-9% of the added ^{125} I IGFBP-3 binds to cell monolayers of fibroblast and rat glioma cells (Yang *et al.*, 1996). Therefore, the use of the correct experimental conditions might be crucial to the success of the experiment.

One of the major technical problems was that the background binding of IGFBP-2 to plastic plates or tubes was higher in the absence than in the presence of cell membrane or ECM preparations. Therefore,

although specific binding of recombinant IGFBP-2 to cell membrane/ECM could possibly occur, it would be undetectable, as it would be masked by the high background. Despite this high background problem, when radiolabelled IGFBP-2 was used as a probe, an indication of binding specificity could be deduced if cold IGFBP-2 displaced the tracer in a dose dependent manner. Unfortunately, in most cases the addition of cold IGFBP-2 failed completely to affect tracer binding. This could have happened because IGFBP-2 tracer binding was not specific in the first place or simply because of the presence on the plastic surface of a large amount of unsaturated binding sites, which would sequester cold IGFBP-2 and prevent it from competing the tracer. An alternative explanation may be that the concentration of cold IGFBP-2 estimated to be 10-50 fold higher than tracer concentration might not be sufficient for displacement. Russo *et al.* (1997), showed IGFBP-2 associated to olfactory bulb cell membranes through GAGs. Protein interaction with GAGs can range from high to low affinity and specificity (Kjellen and Lindahl, 1991), but is generally a high capacity type of binding, due to the relative abundance of GAG compared to the ligand. As a consequence, displacement of the IGFBP-2 tracer might require an extremely high concentration of cold protein. As already mentioned in table *3.3.1.a.*, when we incubated Clone 9 and A10 monolayers with ^{125}I IGF-I to test if the method was able to detect specific binding, we obtained tracer binding only slightly higher than the background, but the addition of cold IGF-I successfully competed the tracer.

Many different methods have been used in an attempt to overcome the problem outlined above.

1) The use of positively charged (Primaria) plates has been described to reduce the background binding of IGFBP-5 to plastic (Jones *et al*, 1993a), but it did not appear to resolve the problem in our case (Exp. *3.3.1.a.4* and *3.3.2.a.8*). Similarly, Parker *et al.* (1996) demonstrated IGFBP-5 binding to fibroblast ECM prepared on Primaria plates, but recently reported that non specific binding of IGFBP-5 to pSM cell ECM coated plates was as high as 50% and consequently they developed an extraction method to overcome the problem (Parker *et al.*, 1998).

2) In order to increase the ratio between cell membranes and plastic surface available for tracer binding we incubated ^{125}I IGFBP-2 tracer with a high density A10 or 3T3F442A cell suspension instead of cell monolayers (Exp. *3.3.1.a.5*). After discarding the radiolabelled IGFBP-2 supernatants, residual radioactivity in plastic tubes did not vary in the presence or in the absence of cell pellets. The addition of cold IGFBP-2 during the incubation of cells with tracer was unable to compete ^{125} I IGFBP-2 binding. In

conclusion, we were unable to demonstrate IGFBP-2 binding to the plasma membrane of A10 and 3T3F442A cells although we cannot exclude that the binding did occur.

3) Saturation of cell surface binding sites by naturally produced IGFBP-2 could be responsible for the failure of exogenous IGFBP-2 to bind to the cell surface. Therefore we tried to use a cell line which does not produce IGFBP-2 (Exp.*3.3.1.a.5*) and, in addition we tried to strip the endogenous protein from plasma membranes of cells that naturally produced IGFBP-2 (Exp. *3.3.1.a.3*).

Previous work carried out in our laboratory demonstrated that in 3T3F442A cell conditioned medium the principal IGFBP detectable by ^{125} I IGF-I ligand blot was a 40kDa protein (which co-migrated with IGFBP-3 bands in mouse serum control), followed by a 24kDa IGFBP (probably IGFBP-4, which co-migrated with a mouse serum control). No proteins co-migrating with the 32kDa IGFBP-2 band of mouse serum were found in 3T3F442A conditioned medium by ligand blotting. Therefore, we considered that these cells could be used as a control cell line to test IGFBP-2 binding to cell membranes in the absence of endogenous protein. Again, the high background of radioactive IGFBP-2 bound to plastic made the results uninterpretable. We cannot exclude the possibility that endogenous IGFBP-3 might be associated with 3T3F442A cell membranes and compete with IGFBP-2 binding. This would imply that IGFBP binding to cell membranes is not specific. Testing this hypothesis was beyond our purpose and we did not pursue the investigation further.

As the only reports of IGFBP-2 association to cell membranes available in literature referred to the detection of the endogenous proteins, in rat olfactory bulbs (Russo *et al.*, 1997), and a small cell lung cancer (SCLC) cell line (Reeve *et al*, 1993), we focused our research on IGFBP-2 producing cell lines. We hypothesised that IGFBP-2 secreting cells might be more likely to express specific IGFBP-2 binding sites on their membranes, though these studies by Russo and Reeve showed that IGFBP-2 secretion by the cells did not necessarily imply its association to the membranes.

To test if IGFBP-2 binding to Clone 9 and A10 cell membranes might be inhibited by endogenous IGFBP-2 that had saturated the available binding sites, we treated the cell monolayers with 20mM sodium acetate, pH4 and 2M NaCl as described by Andress (1995) for stripping IGFBP-5 from mouse osteoblasts. Once more, after the stripping treatment and incubation with ^{125}I IGFBP-2, the amount of tracer bound to the cell monolayer failed to exceed the radioactive background measured in cell-free

wells. No differences were observed between the two cell lines, or when cold IGFBP-2 was co-incubated with the tracer.

Although the results of the first group of experiments (from *3.3.1.a.1.*to *3.3.1.a.5*) were uninterpretable due to the high background binding of ^{125} I IGFBP-2 tracer to plastic, we had evidence (from experiments that will be presented in section *3.3.1.b*) that endogenous IGFBP-2 was associated to cell membranes. Therefore we examined if ^{125} I IGFBP-2 would be better exploited as a probe on protein blots or in cross-linking experiments.

In a preliminary experiment (data not shown), we assessed if the ^{125} I IGFBP-2 ligand blotting technique was suitable for detecting an IGFBP-2 binding molecule by including in the gel an anti IGFBP-2 antibody as a positive control, along with A10 and Clone 9 membrane samples. As the antibody control displayed a faint band at the expected size and the membrane samples also showed a band at the edge of the migration front, we were encouraged to repeat the experiment at a higher % acrylamide gel. We attempted to employ the ^{125}I IGFBP-2 ligand blotting technique to identify a putative IGFBP-2 binding molecule on Clone 9 and MC3T3-E1 cell membranes (Exp. *3.3.1.a.6* and *3.3.1.a.8*), but results were not completely consistent and difficult to interpret. In Fig *3.3.1.a.6* a doublet of approximately 34-36 kDa represents the only band detected by ^{125}I IGFBP-2 in each membrane track. The incubation of the other half of the blot with cold IGFBP-2 failed to compete the tracer (data not shown). In Fig *3.3.1.a.8.***a**, IGFBP-2 tracer detected many protein bands in both concentrated or 0.5% Triton extracted Clone 9 plasma membrane tracks. Moreover, tracer binding was specifically competed by high concentration of cold IGFBP-2 (25ug). Some of the differences between the two experiments can be explained, as the 2 blots are not directly comparable. In fact in Fig *3.3.1.a.6* the membrane proteins were electrophoresed on a 10% gel , while in Fig *3.3.1.a.8* **a**) a 12% gel was used. The 28kDa protein band was possibly absent on the 10% gel due to its small size. Also, it is possible that the 50 kDa band present in the 12% gel might be undetectable in the 10% blot due to the darker background of the film (two different film types were used). In Fig *3.3.1.8* **a** the doublet 32-34 kDa band detected by IGFBP-2 tracer in the membrane samples, was also present on the ECM sample track. It is surprising to note that when the same ECM preparation samples that were used for ^{125}I IGFBP-2 ligand blots, were probed with ^{125}I IGF-I (shown on the right hand side, Fig *3.3.1.a.8* **b**) a very similar picture was produced.

No convincing conclusions can be derived from these experiments.

An interesting interpretation of these results may be that, despite a small difference in electrophoretic migration which could be explained by experimental variability, the 36 or 34kDa bands (single or double), detected with both IGF or IGFBP-2 ligand blotting techniques might represent the same protein/s and, more precisely, endogenous IGFBP/s present in membrane or ECM samples. This would imply that ^{125}I IGFBP-2 binds to blotted IGFBPs (including IGFBP-2). The hypothesis could be tested by including an IGFBP-2 control in the blot, but such a control is not presented as the possibility that IGFBP-2 tracer would bind to cold IGFBP-2 was totally unexpected. Moreover, when IGFBP-2 tracer was incubated with DSS in cross-linking experiments (Exp.*3.3.1.a.7*), no formation of dimer/polymer aggregates was observed.

Nevertheless, the recurrent appearance of 34-36 kDa bands in membrane/ECM samples analysed by either ^{125}I IGF-I or ^{125}I IGFBP-2 ligand blotting (Fig *3.3.1.a.8*) is at least a surprising coincidence.

With regard to the IGFBP-2 cross-linking experiment (Exp. *3.3.1.a.7*), here we would like to explain the rational of the experiment. Sodium chlorate, which is a potent inhibitor of sulphate adenylyltransferase, reduces sulfation of GAGs, without interfering with their synthesis (Hoogewerf *et al.*, 1991). $NaClO_4$ treatment was reported to effectively reduce binding of some proteins to cell surfaces (Hoogewerf *et al.*, 1991, Roghani and Moscatelli, 1992). Treatment of cell monolayers or with $NaClO_4$ or glycanases (heparinase, heparitinase, chondroitinase) are the most commonly used methods used to investigate IGFBP interaction with GAGs. Smith *et al.* (1994) provided some evidence of sodium chlorate inhibitory effects on recombinant IGFBP-3 binding to Sertoli cell membranes. The only work that investigated the nature of IGFBP-2 binding to plasma membranes (Russo *et al.*, 1997) showed that IGFBP-2 binds to rat olfactory bulb cell membranes specifically through chondroitin sulfate and keratan sulfate. Contradictory evidence on IGFBP-2 binding to GAGs *in vitro* have been reported in the literature (Arai *et al.*, 1996 Russo *et al.*, 1997). The ability to bind to heparin and other GAGs *in vitro* has been demonstrated for most IGFBP species (Arai *et al.*, 1994a), and, at least for IGFBP-5, it has been shown that heparinase treatment decreases the ability of the protein to bind to fibroblast ECM (Arai *et al.*, 1996). On the other hand, IGFBP-3 and -5 have been shown to interact with proteins ("receptors") on the cell membrane (Oh *et al.*, 1993b; Andress, 1995). It should also be considered that all membrane GAGs,

except hyaluronic acid, are bound to a core protein to form proteoglycans and protein interaction with membrane proteoglycan can involve the carbohydrate and the protein part, as shown in the binding of thrombin to thrombomodulin (Kjellen and Lindahl, 1991). Therefore, it cannot be excluded that IGFBP-2 association with plasma membranes involves both GAGs and proteins (which may be the protein part of a proteoglycan). We tested possible protein-protein binding by ^{125}I IGFBP-2 cross-linking to Clone 9 membranes. As already mentioned in *3.3.1.a.7*, the DSS cross-linking agent preferentially links NH_2-primary amine groups (Partis *et al.*, 1983) and, as we expected, was unable to covalently link heparin (included as a GAG control) and IGFBP-2 in a complex of expected the Mr 38-40kDa. Instead formation of high Mr aggregates was observed. Although it cannot be excluded that the bands on the top of the gel contained ^{125} I IGFBP-2 cross-linked with a high Mr membrane protein, it is more likely that they are non specific aggregates commonly observed in cross-linking experiments. If sulfation of GAG was relevant for binding, sodium chlorate treatment could decrease the initial ^{125} I IGFBP-2 association to cell membranes whether it was bound or covalently cross-linked to the membranes. However, we believe that the bands shown in Fig *3.3.1.a.7* represent a residue of tracer physically trapped in the pellet or even derived from tube walls irrespective of the presence of the pelleted cells. Based on these results any further speculation on the role of GAG sulfation on the interaction of IGFBP-2 with cell membranes, would be unwarranted. Similarly, the investigation on the possible interaction of IGFBP-2 with integrins remained inconclusive.

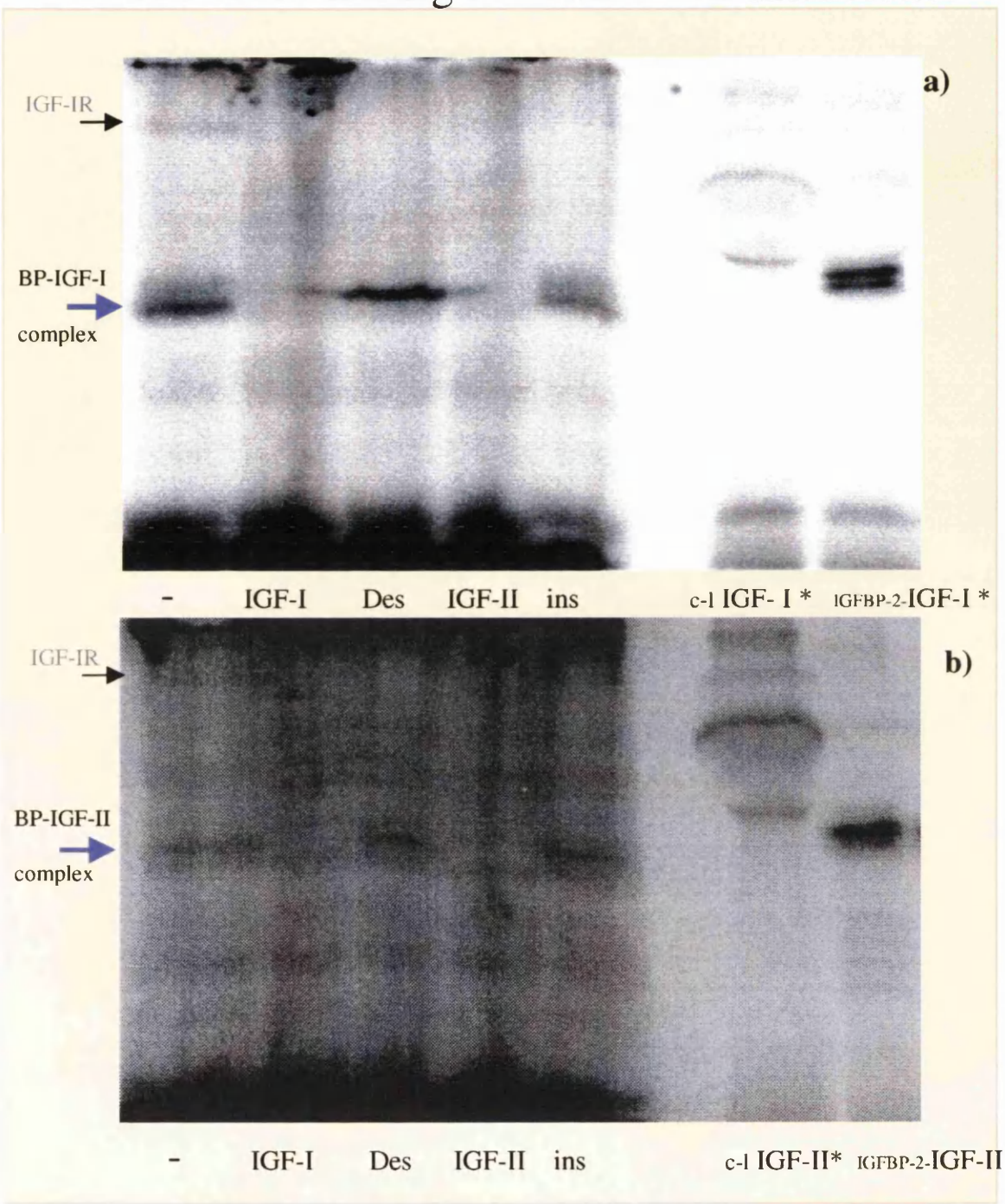
3.3.1.b Detection of endogenous IGFBP-2 associated to the cell surface.

3.3.1.b.1 ^{125}I IGFs cross-linking studies on Clone 9 and MC3T3-E1 membranes.

Most of the evidence on IGFBP-2 binding to cell surfaces reported in the literature has been obtained with an indirect technique, such as cell membrane ^{125}I IGF-I or II affinity labelling and cross-linking (Reeve *et al.*, 1993; Russo *et al.*, 1995; Russo *et al.*, 1997; and recently Bradshaw *et al.*, 1999). We applied the ^{125} I IGF-I and IGF-II cross-linking technique to Clone 9 membranes and Fig *3.3.1.b.1* **a** and **b** show an autoradiograph of affinity labeled complexes formed and electrophoretically separated on a 12% reducing gel. In addition to the size of the complexes, information on the identity of the bands was derived from the competition pattern obtained when cold IGF-I, IGF-II, des(1-3) IGF-I or insulin were included during the cross-linking reaction.

Fig *3.3.1.b.1*

^{125}I-IGF cross-linking to Clone 9 cell membranes



400ug of Clone 9 membranes were cross-linked with ^{125} I IGF-I (Fig **a**) or II (Fig **b**)in the presence or in the absence of 1ug/ml cold IGF-I, des(1-3) IGF-I, IGF-II or 10ug/ml insulin. Control samples of IGF tracer cross linked in the absence (c-l IGF-I, c-l IGF-II) or in the presence of IGFBP-2 were included.
Affinity labeled samples were electrophoresed on a 12% gel under reducing conditions and dried gels were exposed directly to autoradiographs or analyzed with the phospho-imager.

^{125}I IGF-I cross-linking, in the absence of cold competitors, showed a major 36kDa and a 130kDa band. The latter likely represents the complex between tracer and IGF-IR α subunit as it has the expected size and is competed by cold IGF-I, IGF-II, des(1-3)IGF-I and insulin. All these peptides are known to bind to the IGF-I receptor, although IGF-II and insulin have a 10 and 500 fold reduced affinity respectively (Nissley *et al.*, 1991). The major band detected by the tracer, in the absence of competitors, migrated with an apparent Mr of approximately 36kDa and it was accompanied by a fuzzy shadow just above it (38kDa). These bands are believed to represent the complexes formed between radiolabelled IGF-I (7.5 kDa) and cell surface associated IGFBPs. The competition pattern obtained in the presence of cold IGFs, IGF analogue or insulin supports this hypothesis. The appearance of the 38kDa band was inhibited by cold IGF-I and II, but not by insulin, which does not bind to IGFBPs. Des(1-3)IGF-I, which has a 50 fold reduced affinity for IGFBPs (Heding *et al.*, 1996, Oh *et al.*, 1993a), did not compete with radiolabelled IGF-I. Interestingly, in the presence of the IGF-I analogue, the band appeared more intense and defined, and migrated with a slightly higher Mr (similarly to the 38kDa shadow that was evident when the tracer was used in the absence of any competitor or competed by insulin). As a control for the cross-linking reaction, ^{125} I IGF-I was incubated with DSS in the presence or in the absence of recombinant rat IGFBP-2. IGF-I tracer alone did not form any complex (and the only band evident in the lane is the one corresponding to the tracer itself which can also be seen in all other tracks), while the ^{125} I IGF-I-IGFBP-2 cross-linked complex migrated as a double band of about 38-40kDa.

In the ^{125}I IGF-II cross-linking blot, a high background, probably due to the quality of IGF-II tracer, makes it more difficult to distinguish the bands clearly, but a very similar competition pattern is apparent. Again ^{125} I IGF-II cross-linked with a protein of a high Mr (130 kDa), whose appearance was competed by cold IGF-I, -II, des(1-3)IGF-I and partially by insulin. The 36kDa band was the major band detected and it was inhibited by cold IGF-I and -II, but not by insulin and des(1-3)IGF-I. Again des(1-3)IGF-I produced a band shift from 36 to 38 kDa. ^{125} I IGF-II incubated with DSS did not produce any complex, whereas when cross-linked with IGFBP-2 a single 40 kDa band was evident.

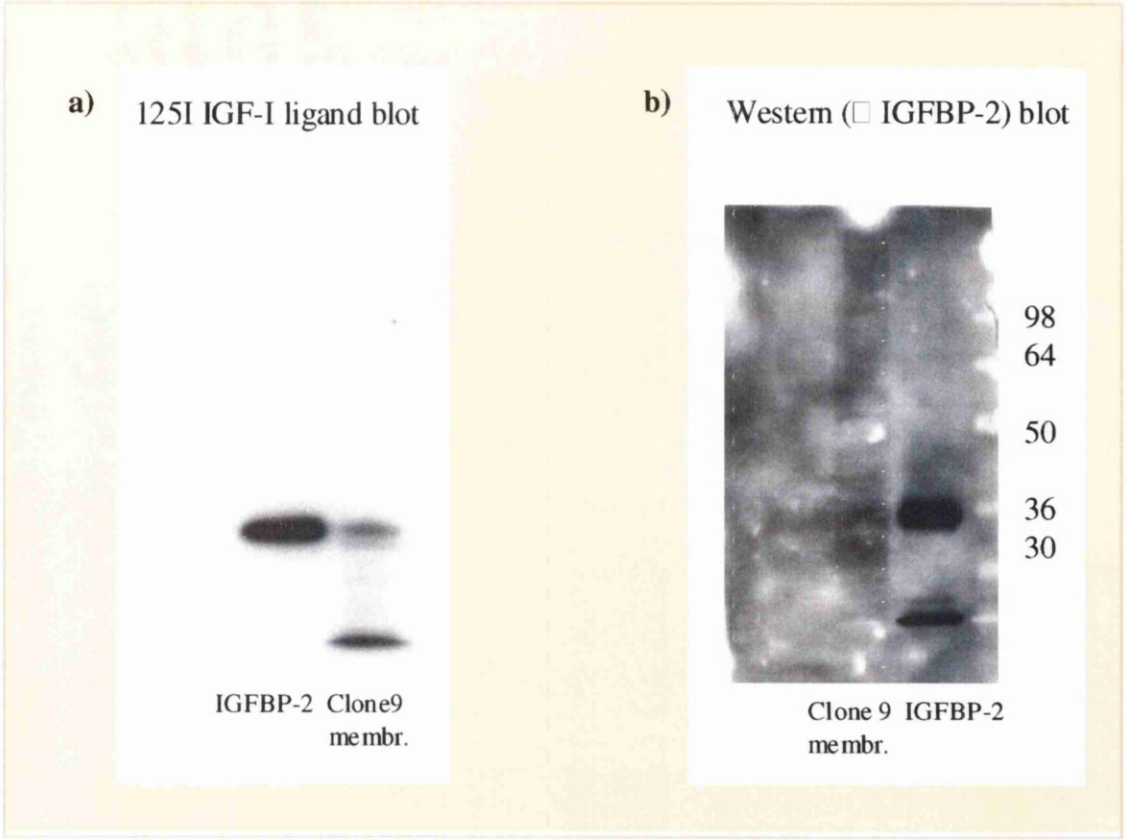
In conclusion, ^{125} I IGF-I and IGF-II cross-linking experiments were able to detect at least one IGFBP (of estimated 30-32 kDa) associated with Clone 9 cell membranes.

3.3.1.b.2 Detection of IGFBP-2 associated to Clone 9 membranes by ^{125}I IGF-I ligand blot and anti IGFBP-2 Western blot.

Proteins in Clone 9 plasma membrane preparations were separated by electrophoresis and transferred to a membrane which was subsequently cut in two halves in order to be differentially processed for ^{125} IGF-I ligand or anti IGFBP-2 Western blot (respectively **a)** and **b)** in Fig *3.3.1.b.2*). ^{125}I IGF-I tracer clearly identified a band with the same electrophoretical characteristics as the recombinant rIGFBP-2 control (34kDa), and another fainter band just below it. The highest radioactivity was associated with the migration front of the gel. The presence of IGF-binding protein species on the cell membranes already shown with cross-linking experiments was confirmed by ligand blotting which does not require chemical cross-linking of ^{125}I IGF-I binding to the membrane. The identification of the binding protein species was first attempted with an anti IGFBP-2 Western blot for two reasons: i) IGFBP-2 was the only binding protein secreted by Clone 9 cells in CM, ii) the protein detected on ^{125} I IGF-I ligand blots showed the same electrophoretical characteristic as the IGFBP-2 control. The anti IGFBP-2 antibody displayed only a diffuse reactivity at 36 and 32kDa in the Clone 9 membranes lane. Fig *3.3.1.b.2***b.2** shows another anti IGFBP-2 western blot on a crude preparation of Clone 9 cells. In this case the band shows a more distinct shape, but it is faint. This figure also shows that the IGFBPs found on the cell surface are not a contamination from soluble binding proteins possibly present in the supernatant.

While comparing ligand and Western blotting autorads at different exposure times, it was noted that the two methods had equivalent reactivity for the IGFBP-2 control, but that the anti IGFBP-2 antibody had a lower sensitivity than the IGF-I tracer for the 36kDa membrane protein. This led us to hypothesise that the image obtained in IGF-I ligand blots could be either due to a totally different IGFBP or it could represent the sum of overlapping IGFBP bands with similar electrophoretic mobility. Excluding IGFBP-3 (a doublet of around 40kDa) and IGFBP-4 (a 24-28 kDa doublet), IGFBP-1, 5, or 6 can all migrate similarly to IGFBP-2 in electrophoresis. As Clone 9 cells are a rat liver cell line and hepatocytes have been reported to secrete principally IGFBP-1 and 2 (Menuelle *et al.*, 1995; Scharf *et al.*, 1995; Hazel *et al.*, 1998), we sought to further analyse the membranes used in ligand blots in an anti rat IGFBP-1 Western blot (data not shown). This antibody, which in a previous experiment was able to recognise IGFBP-1 in the CM of a rat hepatoma cell line (H411E), failed to detect any bands in the Clone 9

Fig.*3.3.1.b.2* Detection of IGFBP-2 on Clone 9 cell membranes

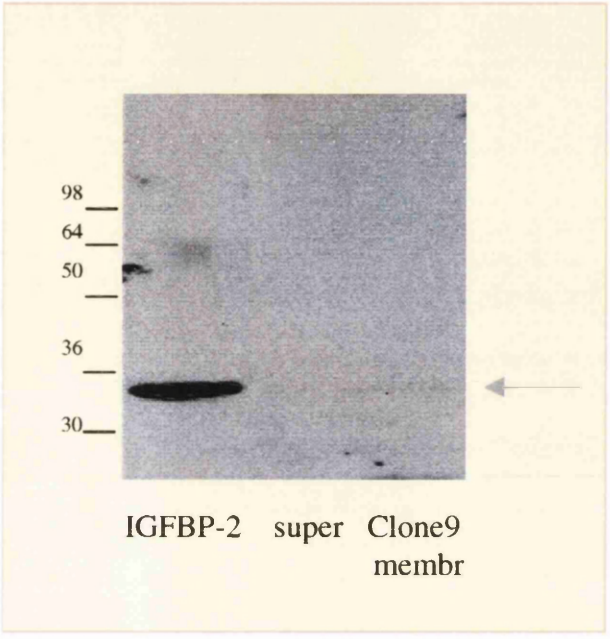


Enriched plasma membrane preparations from Clone 9 cells were obtained as described in Mats & Meths. Samples of the crude preparation were solubilised in 2x Laemmli sample buffer and run in a 12% acrylamide gel under non reducing conditions. After blotting,the membranes were cut in two halves and differently processed for ligand or Western blots following the protocols described in Mats & Meths. Anti-BP2 antibody 1:2000; anti-rabbit-HRP 1:5000.

Fig *3.3.1.b.2.***b.2**

IGFBP-2 associated to Clone 9 cell membrane

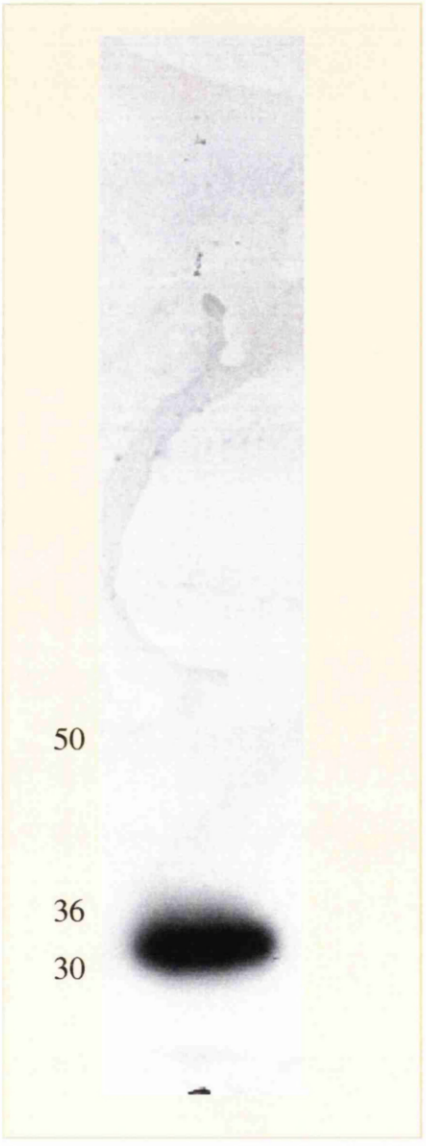
Anti IGFBP-2 Western blot



The picture shows an anti IGFBP-2 western blot of a sample of Clone9 membrane preparation and an equivalent volume of the supernatant derived from the last centrifugation step of membrane preparation. Samples were first electrophoresed on a 12% gel , blotted and then processed for Western blotting. (Anti IGFBP-2 at 1:2000 dilution; anti rabbit-HRP second antibody at 1:5000 dilution)

Fig *3.3.1.b.2.***b.3**

^{125}IGF-I ligand blot of Clone 9 cell conditioned medium



^{125}IGF-I ligand blot of Clone9 cell conditioned medium. The picture shows a single IGF-binding protein species of approximately 32kDa. A smaller fragment (20kDa approx.) is also evident.
(from Dr J. Beattie, with permission)

membrane track, and did not react with the IGFBP-2 control. As will be shown in the following experiments we also tested anti IGFBP-5 with Clone 9 membrane associated binding proteins.

Repeated analysis of Clone 9 cell conditioned medium by ligand blotting over a period of several years in our laboratory has revealed only one major binding protein species at Mr of approximately 32kDa (Fig *3.3.1.b.***2.c**). This band has never presented as a doublet and this protein corresponds to IGFBP-2, as identified by Western blotting (data not shown). In addition, using an "in house" anti-IGFBP-5 antibody we have no conclusive evidence for the presence of IGFBP-5 in medium conditioned by Clone 9 cells.

In conclusion, the presence of Clone 9 membrane-associated IGFBPs was confirmed in ^{125} IGF-I ligand blots. Anti IGFBP-2 Western blots suggested that IGFBP-2 could be present as one of the species.

3.3.1.b.3 Anti IGFBP-2 and 5 immuno-precipitation(IP) of solubilized Clone 9 and MC3T3-E1 membranes. Detection of precipitated proteins by ^{125}I IGF-I ligand blot.

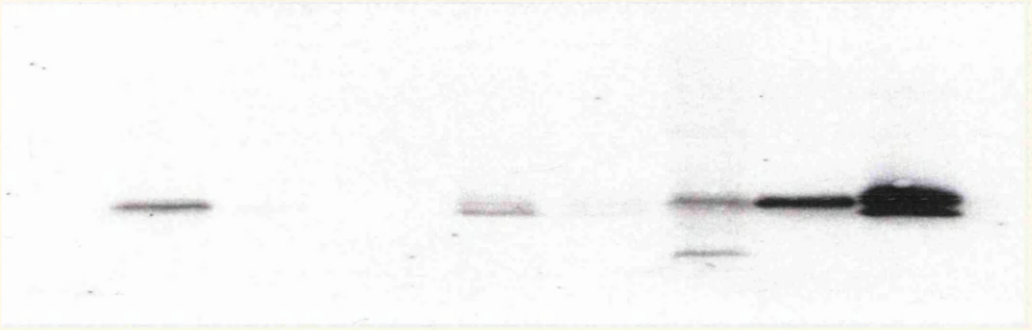
Solubilized Clone 9 and MC3T3-E1 membranes were immuno-precipitated with anti IGFBP-2 or anti IGFBP-5 antibodies and the immune complexes were pelleted with Pansorbin. Immuno precipitated IGFBPs were detected in ^{125}I IGF-I ligand blots (Fig *3.3.1.b.*3.**a** and *3.3.1.b.3.***b**). A sample of crude membrane preparation (i.e. non immune precipitated), and recombinant rIGFBP-2 or rIGFBP-5 were also included. Recombinant rIGFBP-2 (non immune-precipitated), migrated as a single band of 34kDa, recombinant rIGFBP-5 migrated as an equally intense doublet of 33-35kDa. In the literature IGFBP-5 has been reported either as doublet or as a single band (Camacho-Hubner *et al.*, 1992; Tonner *et al.*, 1997). Whether the two bands result from different IGFBP-5 transcripts or postranslational modifications (IGFBP-5 is O glycosylated and phosphorylated) or whether they have a different biological functions is not known.

In the upper autoradiograph (Fig *3.3.1.b.3***a**), Clone 9 membranes (non immune-precipitated) directly analysed by ligand blotting showed a 34kDa band accompanied by a 28kDa band, substantially reproducing the results shown earlier in Fig 3.3.1.b.*2.***a**. After solubilization of the membranes and immuno-precipitation of cell surface-associated IGFBP with anti IGFBP-2 antibodies, a single 34kDa band appeared. This band was more intense than the corresponding band in crude membrane preparations, indicating that the immuno-precipitation had a concentrating effect. Anti IGFBP-5 immune precipitated a doublet formed by a faint upper band (34kDa) and a more abundant band of slightly smaller size. No

Fig *3.3.1.b.3*

IP of solubilised Clone 9 and MC3T3-E1 membranes followed by ^{125} I IGF-I ligand blot

a) Clone 9 cells



SM	Clone9 memb	-	Clone 9 Pansorb	Clone9 memb	_	Clone9 memb	IGFBP-2	IGFBP-5
Ab	αBP-2	αBP-2	_	αBP-5	αBP-5	_	_	_

b) MC3T3-E1 cells

SM	MC memb	-	MC Pansorb	MC memb	_	MC memb	IGFBP-5	IGFBP-2
Ab	αBP-2	αBP-2	_	αBP-5	αBP-5	_	_	_

Solubilised membranes from Clone 9 or MC3T3-E1 cells were immune- precipitated with anti IGFBP-2 or anti IGFBP-5 antibody and pelleted by the addition of Pansorbin. Samples were then run in a 12% gel under non reducing conditions. After blotting, the membranes were processed for an ^{125}I IGF-I ligand blot as described in Mats.& Meths.

bands appeared when Pansorbin was incubated with solubilized membranes without the antibodies, nor when anti IGFBP-2 antibodies were pelleted with Pansorbin, in the absence of membranes. In the track containing anti IGFBP-5 alone a faint reactivity was detectable.

The bottom gel (Fig *3.3.1.b.3.***b**) shows an identical experiment performed on MC3T3-E1 cell membranes used as a control. In this case, the direct ligand blot on the crude plasma membrane preparation detected two faint bands at 34kDa and 30kDa while the darkest band was associated with the migration front of the gel. Anti IGFBP-2 antibody was essentially unable to immuno-precipitate any proteins from solubilised MC3T3-E1 plasma membranes, nor showed any reaction when precipitated with Pansorbin in the absence of membranes. However, when anti IGFBP-5 antibody was incubated with solubilised MC3T3-E1 membranes, it was able to precipitate a doublet which co-migrated with the smallest band of the IGFBP-5 control and resembled the doublet immunoprecipitated from Clone 9 membranes. However the same, but much less intense, doublet band showed when anti IGFBP-5 antibody was precipitated with Pansorbin in the absence of membranes, indicating that perhaps some binding proteins were present in this antibody preparation and led to a background activity. Nevertheless, the presence of Clone 9 or MC3T3-E1 membranes had a distinct effect on the amount of binding protein immuno-precipitated by anti IGFBP-5 antiserum.

In conclusion by anti IGFBP-2 immune precipitation of solubilized Clone 9 membranes we were able to determine, at least, in part the identity of membrane associated IGFBPs. The same experiment performed with anti IGFBP-5 antibody was more equivocal although it suggests that more than one IGFBP may be present on the surface of Clone 9 cells.

3.3.1.b.4 DISCUSSION

In this section we presented the results of studies undertaken to detect endogenous IGFBP-2 associated with Clone 9 cell membranes.

First, we affinity labelled Clone 9 cell membranes with ^{125}I IGF (cross-linking Exp *3.3.1.b.1*), as it seemed to be a well reported method for studies on membrane-bound IGFBP-2. In addition, this technique provides a general picture of all IGF-binding sites available on the cell surface, including IGF-IR, IGF-IIR, insulin R and IGFBPs.

The figures shown (Fig *3.3.1.b.1* **a** and **b**) are representative of a series of cross-linking studies we undertook on Clone 9 or on MC3T3-E1 cells. While IGF receptors were always easily revealed, detection of cross-linked complex between IGF tracer and cell associated IGFBPs was only shown distinctively by using membrane preparations that were 5 times more concentrated than that reported in the literature (80-100μg/μl). In both IGF-I and IGF-II cross-linking experiments, a 36-38kDa complex was apparent. Although the band migrated slightly faster than theoretically expected (Mr of 38-40kDa), the competition pattern obtained in the presence of cold des (1-3)IGF-I and insulin indicated that the complex was indeed formed by the tracer and an IGFBP. When a high concentration (10μg/ml) of insulin was added (which has low affinity for IGF-IR and does not bind to IGFBPs), it competed with ^{125}I IGF binding to IGF-IR, but it did not displace the tracer from the 36-38kDa band. Similarly, des(1-3)IGF-I, which has normal affinity for IGF-IR but reduced affinity for IGFBPs, competed IGF-I/II tracer binding to IGF-IR, but not to IGFBP. However, unlike insulin, des IGF-I seemed to cause a small upward shift of the lowest part of the IGF-IGFBP complex so that the band migrated as a single 38kDa species. The protein shift could be simply due to a technical artifact with no biological meaning or it might be an indication of the multiple nature of the 36-38kDa protein species. Des(1-3)IGF-I has reduced affinity for IGFBPs, but it still retains a residual binding capacity that can vary for different IGFBPs (Oh *et al.*, 1993 a, Mohan *et al.*, 1995; Heding *et al.*, 1996). Therefore, if the 36 and 38kDa bands represented two different membrane associated IGFBPs, it is possible that des(1-3)IGF-I was able to bind to the smaller IGFBP, while it was unable to displace the tracer from the larger IGFBP.

Not only was the cross-linking technique useful in detecting membrane associated IGFBPs, but, as already mentioned, it also provided information on the IGF receptor profile in Clone 9 cells. In all experiments carried out in both Clone 9 and MC3T3-E1 cells, a 130kDa band appeared (Fig *3.3.1.b.1.***a**), which, as confirmed by the competition pattern, represented the α subunit of the IGF-IR. Conversely, the identification of the high Mr radioactive band at the top of gel is not straight forward as its size and the competition pattern are not clearly detectable. In a previous experiment, where Clone 9 membranes were ^{125}I IGF-I cross-linked and electrophoresed on a 7.5 % reducing gel, the Mr of this band was estimated to be approximately 270kDa (data not shown), but unfortunately no IGF-II tracer or cold IGF-II were used at that time. A comparison between the competition patterns of IGF-I and IGF-II cross-linking experiments

would be very informative as the two tracers have different affinities for the IGF-IIR. Mr estimates for the IGF-IIR ranges between 260 kDa (Sepp-Lorenzino, 1998) and 215kDa, this latter being reported for IGF-IIR in Clone 9 cells (Matovcik *et al.*, 1990). Therefore the 270kDa band we detected in ^{125}I IGF-I cross-linking experiments might be too large (even after subtraction of 7.5kDa for tracer Mr) to coincide with this IGF-IIR description. It is possible that this band was a 270kDa dimer of two IGF-IR α subunits as reported by Bradshaw *et al.* (1999).

The identity of Clone 9 membrane-associated IGFBPs was, at least in part, clarified with immunoprecipitation experiments. Anti IGFBP-2 antibody incubated with solubilized Clone 9 membranes precipitated a protein, which displayed the same electrophoretic characteristics as recombinant rIGFBP-2 and was able to bind ^{125}I IGF-I in ligand blots. When the immune precipitation was carried out with anti IGFBP-5 antisera a doublet appeared, with the upper band having the same Mr as IGFBP-2 and the lower band slightly smaller. As already noted in paragraph *3.3.1.b.2*, the same double band appeared faintly in the anti IGFBP-5 alone track, in the absence of Clone 9/MC3T3-E1 membranes. Although the amount of immunoprecipitated IGFBP indisputably increased in the presence of membranes, suggesting that an additional source of immunoprecipitable IGFBP-5 had been provided, it cannot be excluded that membrane components might simply facilitate the precipitation of immunocomplexes already present in the serum. However, the presence on Clone 9 membranes of an IGFBP, smaller and less abundant than IGFBP-2 seems consistent with the pattern of membrane-associated IGFBPs detected in Fig *3.3.1.b.2.***a**. Moreover the double band detected in ^{125} IGF-I ligand blots with the ECM preparation of Clone 9 cells seems to support also the presence of two IGF binding species.

Lastly, though our results show quite clearly the presence of IGFBP-2, and possibly IGFBP-5, on Clone 9 cell membranes they do not directly prove that the binding proteins are chemically bound to the membranes. Plasma membranes were prepared using a method described in the literature for this type of study. Contamination from CM can be excluded as the cell plates were washed three times before cell lysis and scraping. In addition, no IGFBPs were revealed in the supernatant from the membrane pellet after the last centrifugation using Western blotting. It is a different matter to prove that IGFBP-2 was chemically bound to the membranes and not simply physically associated with it. The first section of experiments where ^{125} I IGFBP-2 was used as a probe to detect membrane binding, could have clarified

this point, but unfortunately the high background binding of the tracer to plastic rendered the results uninterpretable. It may be possible with further research to identify the binding molecule(s) for IGFBP-2 in Clone 9 cells. As already mentioned, Russo and co-workers (1997) reported that IGFBP-2 in rat olfactory bulbs binds to cell surface GAGs, mainly chondroitin and kearatan sulphate, which are the most abundantly expressed GAGs on these membranes. First, they demonstrated IGFBP-2 bound to purified GAGs *in vitro*. Then, they showed that in solubilised olfactory bulb membranes, an anti IGFBP-2 antibody was able to co-immune-precipitate IGFBP-2 associated to a 200kDa molecule, which was recognized by anti chondroitin sulphate antibodies in Western blotting. As we had no information on the nature of the GAGs present on Clone 9 membranes, we tried to visualize any coimmunoprecipitated protein by reprobing the ^{125} I IGF-I ligand blot shown in Fig *3.3.1.b.2* **a** and **b** with ^{125} I IGFBP-2. Unfortunately, the only band that appeared was the 150 kDa anti IGFBP-2 antibody (data not shown). Negative results could be due to the inability of ^{125}I IGFBP-2 to bind to any co-precipitated protein in ligand blots. Alternatively, the conditions (1% Triton, 1% Na cholate) used to solubilize Clone 9 membranes before the incubation with anti IGFBP-2 may lead to the dissociation of IGFBP-2 from its cognate membrane binder.

In conclusion, in this experimental section we demonstrated that IGFBP-2 is associated with Clone 9 cell membranes and we provided some evidence that IGFBP-5 may also be present. In addition, we showed that Clone 9 cells express IGF-IR.

3.3.2 Clone 9 cell responsiveness to IGF stimulation.

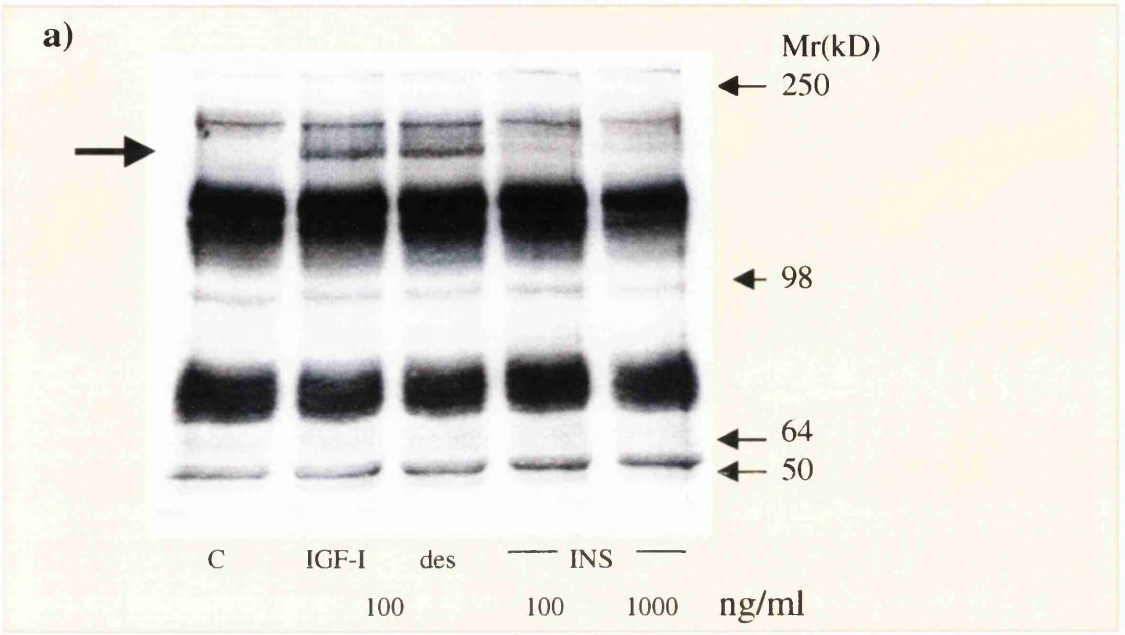
Once we had demonstrated the association of IGFBP-2 to Clone 9 cell membranes, we needed to establish whether Clone 9 cells were a suitable model for investigating IGFBP-2 modulation of IGF action. The first requirement was to demonstrate cell responsiveness to IGFs. Therefore we developed both a short term (signal transduction events) and a long term (MTT assay) assay to show IGF responsiveness of Clone 9 cells.

3.3.2.1. Signalling studies

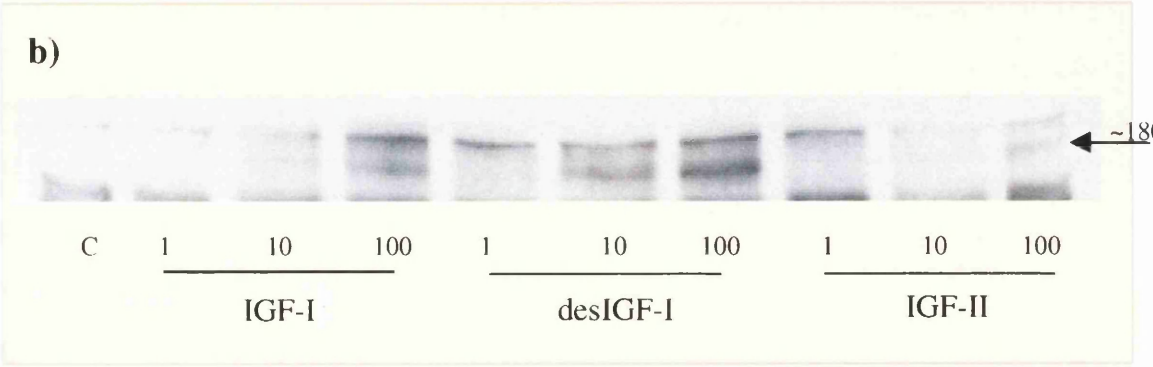
The aim of our signalling studies was simply to identify a measurable effect triggered by IGF stimulation. As one of the earliest events that follows the interaction of growth factors with their receptors

Fig *3.3.2.1.*

Tyrosine phosphorylation induced by IGF treatment in Clone 9 cells



Clone 9 cells were plated in 10 cm dishes and cultured as described in Mats &Meths. After 10min treatment with IGF-I, des(1-3)IGF-I and insulin at the indicated concentrations, cells were lysed and samples (normalized for protein concentration) were run in a 7.5% acrylamide gel. After blotting, membranes were probed with an anti P-tyrosine monoclonal Ab. (4G10, antiPY MAb, at 1: 2000 dilution, anti mouse-HRP 1:4000)



Anti P-tyrosine Western blot of Clone 9 cells treated for 2min with 1, 10, 100 ng/ml of IGF-I, des (1-3)IGF-I and IGF-II. (4G10 diluted 1:1000, anti mouse-HRP diluted 1:1000)

is protein phosphorylation of the receptor and cytoplasmic protein substrates, we tested the effect of a 2-10min stimulation of Clone 9 cells with IGF-I, IGF-II, des(1-3)IGF-I or insulin.

In the upper anti-phospho-tyrosine Western blot (Fig.*3.3.2.*1.**a**), Clone9 cells were stimulated for 10min with a high (100 ng/ml) concentration of IGF-I or des(1-3)IGF-I or with (100 and 1000ng/ml) insulin. The figure shows very clearly that IGF-I and des(1-3)IGF-I treatment induced the appearance of a tyrosine phosphorylated protein with a Mr of approximately 180kDa. This band was absent in the control lane and it was just detectable in insulin treated cells, surprisingly with no differences whether 100 or 1000ng/ml insulin was used. Under our experimental conditions, IGF-I, des(1-3)IGF-I or insulin treatments did not seem to stimulate tyrosine phosphorylation of any other protein, as determined by Western blots.

We then designed a dose-response experiment, in which Clone 9 cells were stimulated for 2min with 1-10-100ng/ml of IGF-I, IGF-II, and des(1-3)IGF-I. Results are shown in Fig *3.3.2.1***b**. It can be observed that the phosphorylated band was not apparent after stimulation of Clone 9 cells with 1 and 10 ng/ml IGF-I and IGF-II, but both peptides used at 100ng/ml induced phosphorylation of the 180kDa protein. Conversely, 10ng/ml of des(1-3)IGF-I were sufficient to stimulate tyrosine phosphorylation of this protein, an effect which seems to increase at 100ng/ml. We noticed that anti-phosphotyrosine Western blotting could occasionally be influenced by non-homogeneous reactivity of the ECL reagents in different areas of the membrane blots. Therefore it might be misleading to draw definitive conclusions on relative potencies of IGF-I and II at 100ng/ml or to exclude that IGF-I could have some effects also at 10ng/ml.

The identity of the 180kDa phosphorylated protein remains unknown. We postulated it could represent the activated form of IRS-I, because one of the earliest events that follow IGF-IR stimulation by IGF-I is the phosphorylation of the 165-185kDa insulin receptor substrate protein, IRS-I (De Meyts *et al.*, 1994; Rubin and Baserga, 1995). Therefore, we tested this hypothesis by anti IRS-I Western blotting (data not shown). Lysate samples from Clone 9 cells, treated +/- 10ng/ml IGF-I, II or des (1-3)IGF-I were electrophoresed. Unfortunately using a rabbit polyclonal anti-rat IRS-I antibody we were unable to detect a protein of the expected size (165-185kDa). Instead a 95kDa band appeared in all tracks (data not shown).

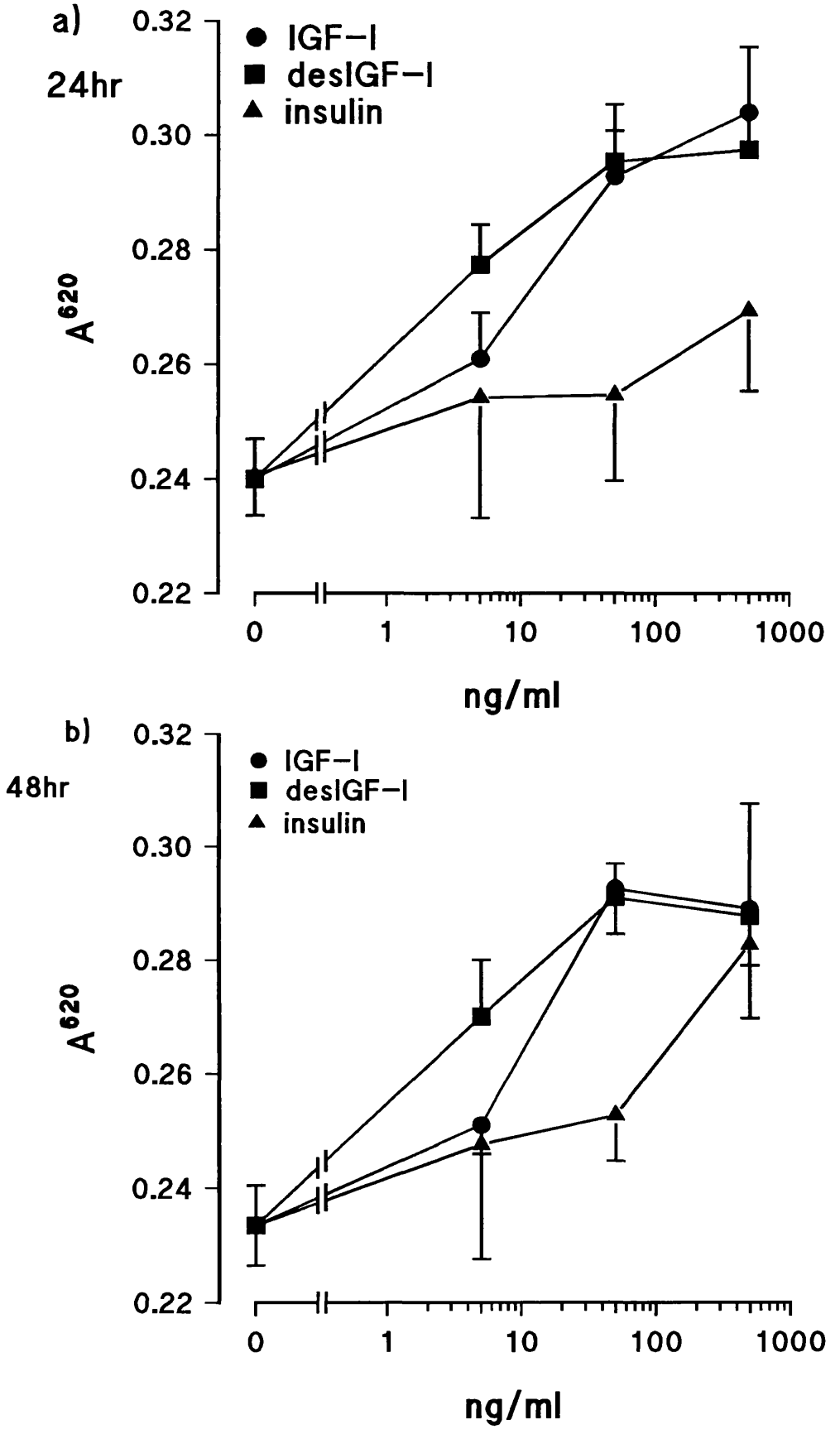
In conclusion, we found that Clone 9 cell responsiveness to acute IGF treatment could be revealed by the induction of tyrosine phosphorylation of a 180kDa protein species.

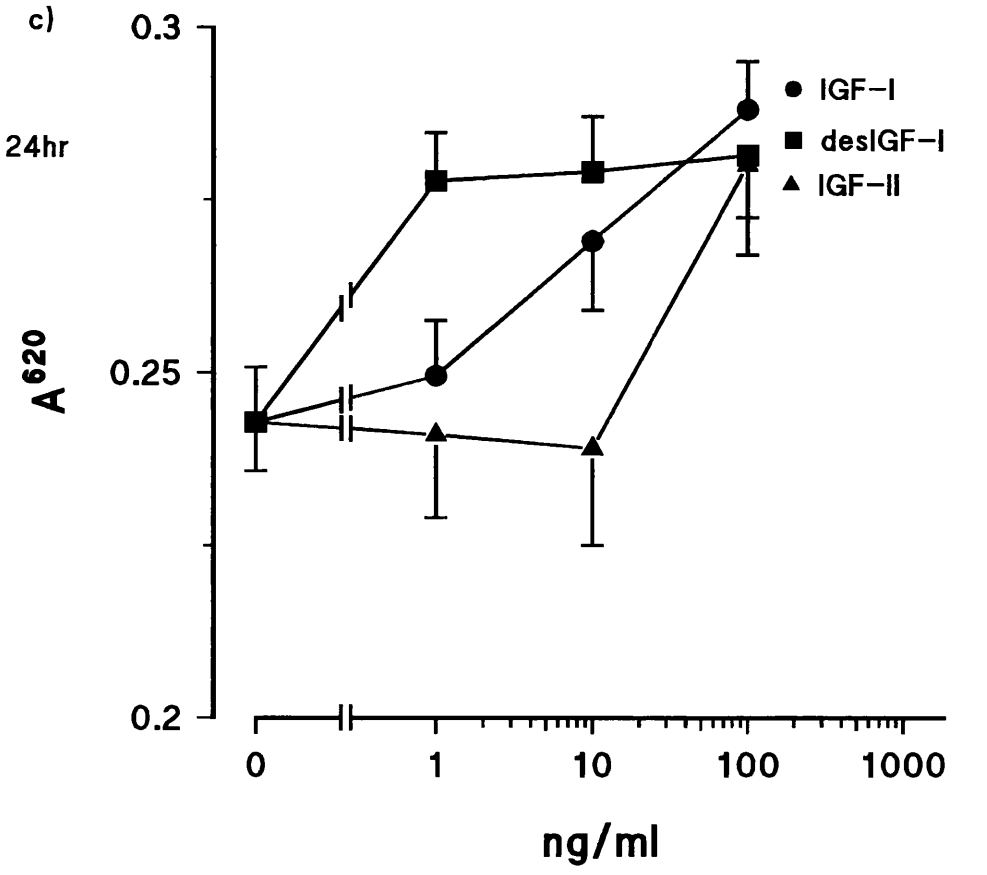
3.3.2.2. MTT assay

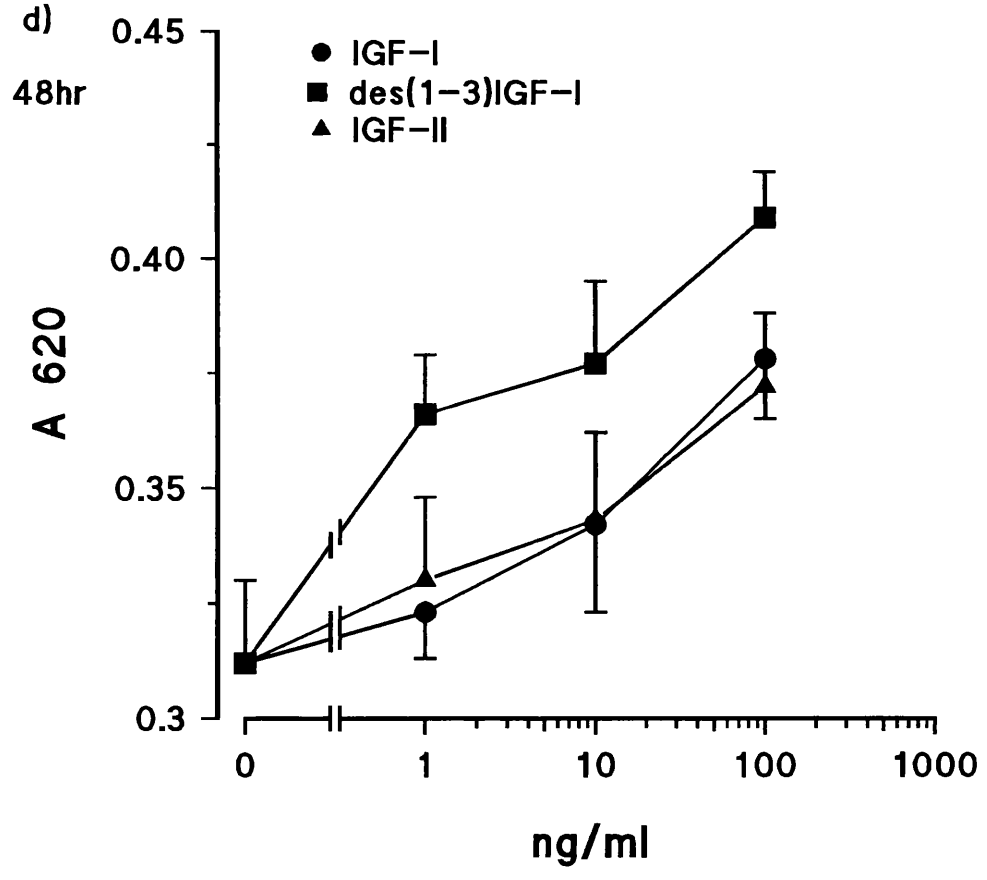
In order to identify other parameters located down stream in the IGF signalling cascade, we tested if the MTT assay, a metabolic/mitogenic bioassay, was exploitable for measuring Clone 9 cell responsiveness to IGF treatment. As explained in the Materials and Methods section the MTT assay measures the amount of yellow MTT, tetrazolium salt, which is transformed to an insoluble, purple, formazan compound by metabolically active cells. In Fig *3.3.2.2.***a** the effects of 24h incubations of Clone 9 cells with 5, 50, 500ng/ml of IGF-I, IGF-II or insulin are presented. 8 replicates for no-treatment controls and for each dose of hormone treatment were measured. Hormone concentrations are indicated on the X axis and the absorbance at 620nm on the Y axis. Each plotted point represents the mean value and the standard deviation. In the first experiment it appeared that both IGF-I and des(1-3)IGF-I were potent cell stimulators, both active at 5ng/ml. At 50ng/ml their effect increased and it reached a plateau at 500ng/ml. Insulin was less potent and did not reach the stimulation level of the other two growth factors even at 500ng/ml. In Fig *3.3.2.2.***b** are presented the results of an identical experiment, except that cells were allowed to grow for 48h. The picture is similar to that at 24hr, except for a higher activity for insulin, which at 500ng/ml, showed the same potency as IGF-I and des(1-3)IGF-I.

Fig *3.3.2.2.***c** shows a 24 h MTT assay in Clone 9 cells treated with 1, 10 and 100ng/ml of IGF-I, des(1-3)IGF-I or IGF-II. In this experiment, insulin treatment was substituted with IGF-II as the direct comparison between the IGFs, and the IGF-I analogue, was more relevant for our research. We intended to test if the apparent difference in cell responsiveness observed between IGF-I and des(1-3)IGF-I treatment at 5ng/ml was amplified at lower treatment concentrations (1ng/ml). It should be noted that in our MTT assay, after hormonal treatment, cells are cultured for 24-48 h. During this time cells grow and produce IGFBPs that, accumulating in the CM, can inhibit cell growth stimulated by IGF, but not by des(1-3)IGF-I as it has almost no affinity for IGFBP. We considered that 5ng/ml of IGF-I (see Fig.*3.3.2.2.***a**) might be almost sufficient to saturate IGFBP binding capacity and overcome their inhibitory effect. Indeed, in the 24h assay, IGF-I and des(1-3)IGF-I and IGF-II potency at 1ng/ml varied remarkably, des(1-3)IGF-I being the most potent, followed by IGF-I and finally IGF-II. In particular the difference between IGF-I and des (1-3)IGF-I hormone treatment resulted in statistically highly significant results. The results of the MTT assay *for the two treatments IGF-I and des(1-3)IGF-I at concentrations of 1ng/ml*

Fig 3.3.2.2 MTT assay on Clone 9 cells




c)
24hr
0.3
0.25
0.2
A^{620}
IGF-I
desIGF-I
IGF-II
0
1
10
100
1000
ng/ml


d)
48hr
0.45
0.40
0.35
0.3
A 620
IGF-I
des(1-3)IGF-I
IGF-II
0
1
10
100
1000
ng/ml

and 10ng/ml were analysed with MINITAB version 11.*21* using *a 2 way ANOVA with replications.* The interaction between the hormones and the concentrations was significant (P=0.007*),* indicating that *the difference between the two treatments is dependent on the concentration.* We then performed an *independent sample t-test to compare* the effect of IGF-I and des(1-3)IGF-I *at each of the two* concentrations. The metabolic/mitogenic effect of the two hormones on Clone 9 cells, as determined with the MTT assay, *was significantly different* at the concentration of 1ng/ml (P<0.001), but not at 10ng/ml (P=0.071).

IGF-II was less efficient than IGF-I in stimulating Clone 9 cells. This was not surprising as IGF-II (compared to IGF-I) has at least 10 times lower affinity for the IGF-IR (Nissley *et al.*, 1991). Moreover, IGFBP-2 has been reported to have higher affinity for IGF-II than IGF-I (Rosenfeld *et al.*, 1989; Roghani *et al.*, 1991; Oh *et al.,* 1993a). As a consequence IGF-II may be sequestered by IGFBP-2 more efficiently and thus interact less with the IGF-IR.

Fig *3.3.2.2.***d** shows a 48h MTT assay in which Clone 9 cells were treated with 1-10-100ng/ml IGF-I, IGF-II, des(1-3)IGF-I. Again des(1-3)IGF-I was the most potent peptide in stimulating Clone 9 cells at all concentrations tested. In this experiment the effect of IGF-I or IGF-II treatment were indistinguishable. Both peptides showed lower potency than des(1-3)IGF-I at each concentration tested. In this case the *2 way ANOVA test with replication* on IGF-I and des(1-3)IGF-I at 1 and 10ng/ml showed that the interaction between hormones and the concentrations was insignificant (P=0.542), which indicates that the difference between desIGF-I and IGF-I was the same no matter what the concentration was. This analysis also indicates that the two hormones were different (P<0.001) and that they stimulated Clone 9 cell growth in a concentration dependent fashion (P=0.010 for concentration effect).

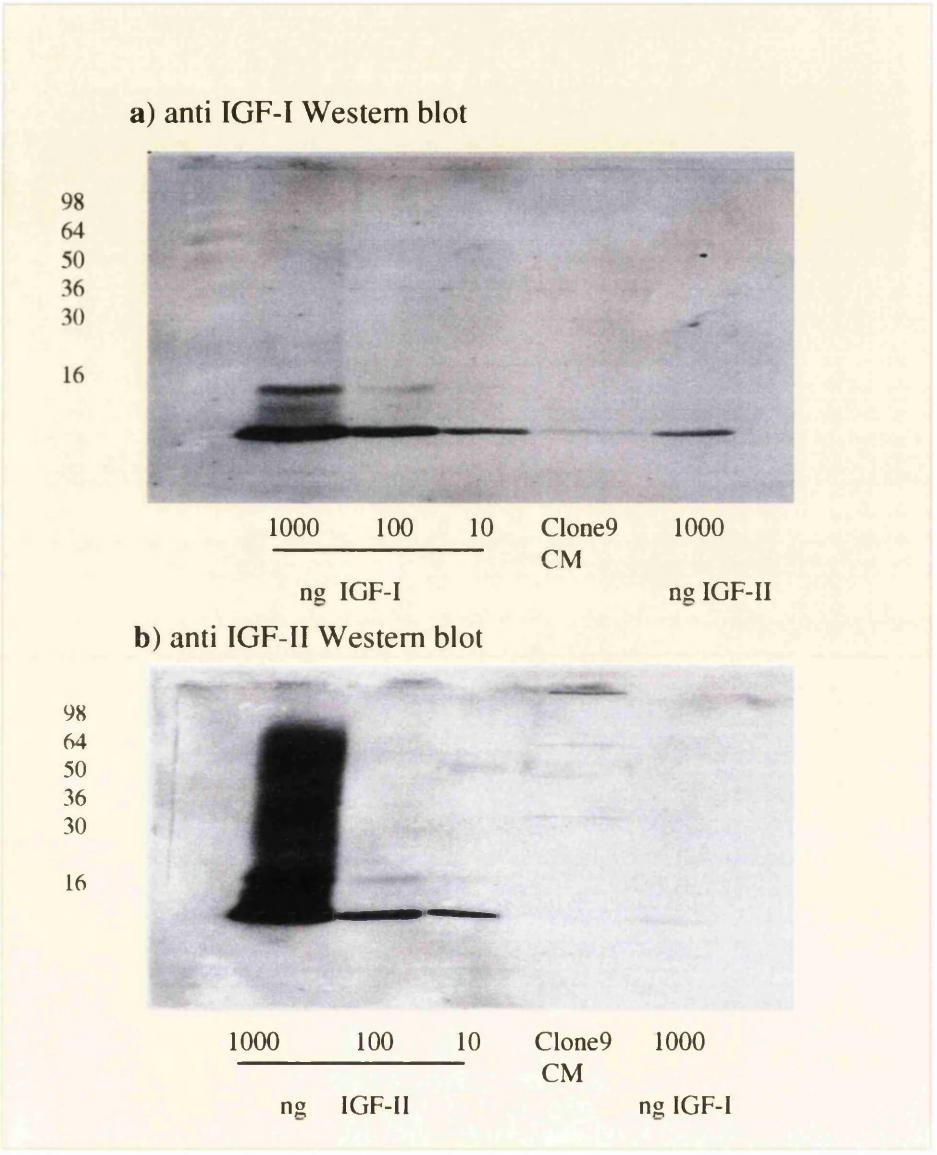
In conclusion Clone 9 cell responsiveness to IGFs can be conveniently measured by 24-48h MTT bioassay. The difference between IGF-I and des(1-3)IGF-I treatments can be usefully exploited to derive the role of IGFBPs in modulating IGF action.

3.3.2.3 Characterisation of IGF production in Clone 9 cells

Samples of 48h concentrated CM from Clone 9 cultures were electrophoresed on 16% acrylamide gels under non reducing conditions, blotted, and analysed by anti-IGF-I and anti-IGF-II Western blotting (Fig *3.3.2.3***a** and *3.3.2.3.***b**). In the (upper) anti IGF-I Western blot, a single band, which showed identical

Fig *3.3.2.3.*

Clone 9 cells secrete IGF-I



Freeze- dried, dialysed and concentrated conditioned medium from Clone 9 cells was run in a 16% acrylamide gel under non reducing conditions. Increasing amounts of standard IGF-I and II were also included in order to check the cross reactivity of the antibodies. After blotting, the membranes were processed for Western blotting using either α IGF-I (fig **a**) or αIGF-II (fig **b**) as the first antibody .

migration characteristics as the hIGF-I standard, appeared in the Clone 9 CM lane. In the lanes where IGF-I was run at high concentration, a 14kDa Mr band was evident and may represent a peptide dimer. However, anti IGF-I antibodies clearly cross-reacted with hIGF-II. Therefore, we tested whether the band detected in Clone 9 CM was actually IGF-I or IGF-II with an anti IGF-II Western blot. Anti-hIGF-II polyclonal antibody, which does not cross react with human or rat IGF-I (<1%), specifically detected 10-100-1000 ng hIGF-II but did not recognise any bands in the IGF-I control or in Clone 9 CM lanes. Combining the results of the two Western blots, we conclude that Clone 9 cells secrete IGF-I, but not IGF-II.

3.3.2.4 DISCUSSION

The results presented in this section represent the preliminary steps necessary to establish a cell model in which the modulation of IGF action by IGFBP-2 could be studied. Our cross-linking experiments demonstrated that Clone 9 cells express IGF-IR and by Western blotting it was demonstrated that the cells secrete IGF-I in the conditioned medium. The presence of most of the elements of the IGF axis (IGF-I, IGFBP/s, IGF-IR and, as reported in the literature, IGF-IIR) indicated that the growth factor might play a functional role in the Clone 9 cell line. We found that these cells were responsive to IGF and that anti phospho-tyrosine Western blotting and MTT assays were suitable methods to measure the effect of treatments. Some differences between the two methods should be highlighted.

Protein phosphorylation (paragraph *3.3.2.1*) in response to IGF signalling is an acute event, which follows a few minutes incubation with hormone and it is principally influenced by the affinity/capacity of the binding sites available on the cell monolayers and the availability of signalling proteins in the cells. Before hormone treatment, cell plates are washed so that the only IGFBPs present are those associated with the monolayer. In Clone 9 cells, des(1-3)IGF-I, at 10ng/ml, appeared to be more potent than IGF-I in stimulating the phosphorylation of a 180kDa protein species (Fig. *3.3.2.1.***b**). This difference between the two growth factors, which have equal affinity for IGF-IR, might be attributed to the inhibitory effect that monolayer associated IGFBPs exert on IGF-I but not on des(1-3)IGF-I. The lesser response of Clone 9 cells to IGF-II treatment in comparison with IGF-I and des(1-3)IGF-I can be explained by its reduced affinity for the IGF-IR. In addition, although our charcoal-binding assay data

disagree, it has been reported that IGFBP-2 affinity for IGF-II is higher than its affinity for IGF-I, therefore IGFBPs present on the monolayer could sequester IGF-II from IGF-IR more efficiently.

Unlike anti phospho-tyrosine Western blotting, the MTT assay (paragraph *3.3.2.2*) measures a long term effect of IGF stimulation. After hormonal treatment, Clone 9 cells are grown for 24-48h and their metabolic-mitogenic response is directly assessed, without the intervention of any washing step. As a consequence, nutrient concentration in the CM will be gradually reduced, while cell metabolism products will accumulate, including secreted IGFBP-2. In this assay, the higher potency displayed by des(1-3)IGF-I in comparison to IGF-I analogue might be due to the inhibitory effect exerted on IGF-I by the total amount of soluble and monolayer associated IGFBPs. Although this assay cannot discriminate between the components of the IGFBP population, it has the advantage of ease, reliability and ability to detect an IGF effect at concentration as low as 1ng/ml.

Some aspects of the MTT assays require elaboration. In both the 24h and 48h MTT assays (Exp. *3.3.2.2.***a, b, c** and **d**), at low concentrations (1-5ng/ml), des(1-3)IGF-I appeared to be more potent than IGF-I in stimulating a metabolic/mitogenic cell response. As already mentioned this could be due to the fact that at these concentrations IGFBP-2 exerts its inhibitory effect on IGF-I but not on des(1-3)IGF-I. On the contrary, in 24h MTT assays (Exp. *3.3.2.2.***a** and **c**) the effect of the two hormones becomes indistinguishable at higher concentrations (10-50-100-500ng/ml). In the two 48hr assays (*3.3.2.2.***b** and *3.3.2.2.***d**) the trend of the cellular response to high doses of hormone stimulation is not consistent. In Exp *3.3.2.2.***b** stimulation of Clone 9 cells with 50-500ng/ml of IGF-I was equivalent to the effect of des(1-3)IGF-I, whereas in Exp *3.3.2.2.***d** IGF-I remained less potent than des(1-3)IGF-I at all concentrations tested, even at 100ng/ml. These differences may be due to experimental variability and more experiments would be required to accurately define Clone 9 cell responsiveness at high hormone doses. However, we focused our experiments on the ability of IGFBP-2 to modulate the effect of IGF-I (but not des(1-3)IGF-I), and were therefore interested in activity at low concentration of hormone.

The final aim of our project was to create a modified Clone 9 cell subclone, whose expression of endogenous IGFBP-2 was impaired by the expression of antisense IGFBP-2 RNA. In this respect we believe that anti-phosphotyrosine Western blotting and the MTT assays would prove to be suitable in order to investigate the effects of IGF treatment in wt and modified cells. In addition, the finding that

Clone 9 cells secrete endogenous IGF-I could be exploited to compare the ability of IGFBP-2 to modulate basal cell growth in wt and antisense transfected cells.

3.3.3 Development of a cellular model to investigate IGFBP-2 biological functions.

Once we had identified the parameters for measuring Clone 9 cell responsiveness to IGFs, we proceeded to isolate a population of modified Clone 9 cells, whose IGFBP-2 expression would be inhibited by using an antisense RNA technique. We intended to compare these modified Clone 9 cells with their wt counterparts, in order to clarify some aspects of IGFBP-2 biological functions. As controls, we transfected Clone 9 cells with sense IGFBP-2 or pcDNA3 vector alone, and we also included sense/antisense IGFBP-5, as we were interested to investigate whether the increased or inhibited expression of IGFBP-2 or 5 could differentially affect cell growth or signal transduction.

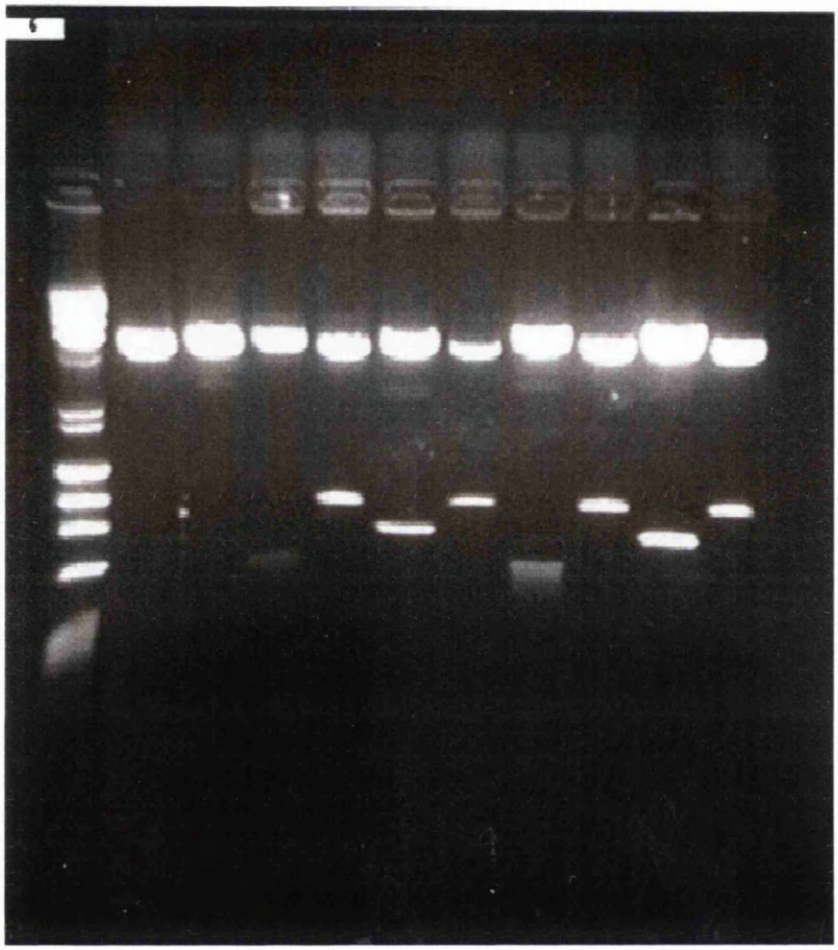
3.3.3.1 Cloning IGFBP-2 and IGFBP-5 into the pcDNA3 expression vector in sense/anti sense orientations.

Fig *3.3.3.1* shows an EcoR1or Hind III restriction digestion of recombinant pcDNA3 expression vector carrying DNAs for IGFBP-2 or IGFBP-5 inserted in either a sense or antisense orientation with respect to the CMV promoter. As IGFBP-2 and IGFBP-5 cDNAs had been cloned into an EcoR1 site in pcDNA3, the digestion of the recombinant vectors with the same endonuclease released inserts of the expected size. Digestion with Hind III was used to check the gene orientation of the insert in the vector. This enzyme cuts the pcDNA3 plasmid once and linearizes it. There is a unique Hind III restriction site at the 5'end of the IGFBP-2 cDNA and also at the 3' end of the IGFBP-5 cDNAs. Therefore it was possible to deduce the orientation of the inserts from the size of released DNA fragments. The expected fragment sizes are summarised in the table shown in Fig *3.3.3.1*

It is clear from the data presented in this picture that we had produced recombinant pcDNA3 plasmids carrying IGFBP-2 or IGFBP-5 cDNAs inserted in either orientation.

Fig *3.3.3.1*

Cloning IGFBP-2 and 5 in the sense/*antisense* orientation into pcDNA3 expression vector



Hind III and EcoR1 single digestions of pcDNA3 vector, pcDNA3-IGFBP-2 sense, pcDNA3-*IGFBP-2antisense*, pcDNA3-IGFBP-5sense, pcDNA3-*IGFBP-5antisense*

EXPECTED FRAGMENT SIZE

	pcDNA	IGFBP-2s	*IGFBP-2a*	*IGFBP-5a*	IGFBP-5s
Hind III	5454	330 6104	750 5684	690 6044	360 5714
EcoR1	5454	980	980	950	950

3.3.3.2 Preliminary experiments to evaluate the growth rate in Clone 9 cells transfected with sense/antisense IGFBP-2 or cDNA3 vector alone.

The growth rate of Clone 9 cells transfected with either sense or antisense IGFBP-2 constructs or with pcDNA3 vector alone was assayed in a time course experiment under basal conditions (i.e. in the absence of exogenous growth factors. For each cell line, the averaged values of 24 replicates and standard deviations at each time point were plotted (Fig *3.3.3.2*). On the X axis is indicated the time (from 0 to 72 hr at 12hour intervals), and on the Y axis is expressed the absorbance measured at 620nm. After an initial recovery phase, the three cell lines, show distinct time-dependent growth. We took into account that the initial density of the cell suspensions used for plating could be slightly different for each of the Clone 9 transfected lines. For this reason, we established the MTT reactivity of each cell line just after cell attachment to the plates (time 0), and we aimed to calculate the individual growth rates rather than comparing the absolute values obtained at each time point. Statistical analysis of the data was performed with GENSTAT 5 version 4.1. using *multiple linear regression with groups*. The shape of the growth curves was not significantly different (i.e. they were parallel) in the 3 cell populations of Clone 9 cells (transfected with sense/antisense IGFBP-2 or pcDNA vector alone) indicating that the growth rate of the cells was not specifically affected by the DNA constructs used for transfection.

In conclusion, in this preliminary time-course experiment, sense/antisense-IGFBP-2 or pcDNA transfected Clone 9 cells, when cultured under basal conditions in 0.1% FBS over a period of 72hr, show the same growth rate (as measured by MTT assay).

3.3.3.3. ^{125} *I IGF-I charcoal binding assay on CM samples from Clone 9 cells transfected with sense/antisense IGFBP-2 or pcDNA vector alone.*

A preliminary assessment of the effect/s that transfection of Clone 9 cells with different constructs had on the expression of IGFBP-2 was carried out using the charcoal binding assay. Equal volumes of serum-free media conditioned for 24h by Clone 9 cells transfected with sense, antisense IGFBP-2 constructs or pcDNA vector alone, were examined for their ^{125}I IGF-I binding capacity. In Fig *3.3.3.3* increasing volumes of CM are indicated on the X axis, while on the Y axis is reported ^{125} I IGF-I binding expressed as a % of the blank value, as described in Materials and Methods (paragraph *2.1.3*). CM from antisense IGFBP-2 transfected cells showed a decreased IGF-binding activity in comparison with

Fig 3.3.3.2Time course MTT assay for sense/antisense pcDNA transfectd Clone 9cells

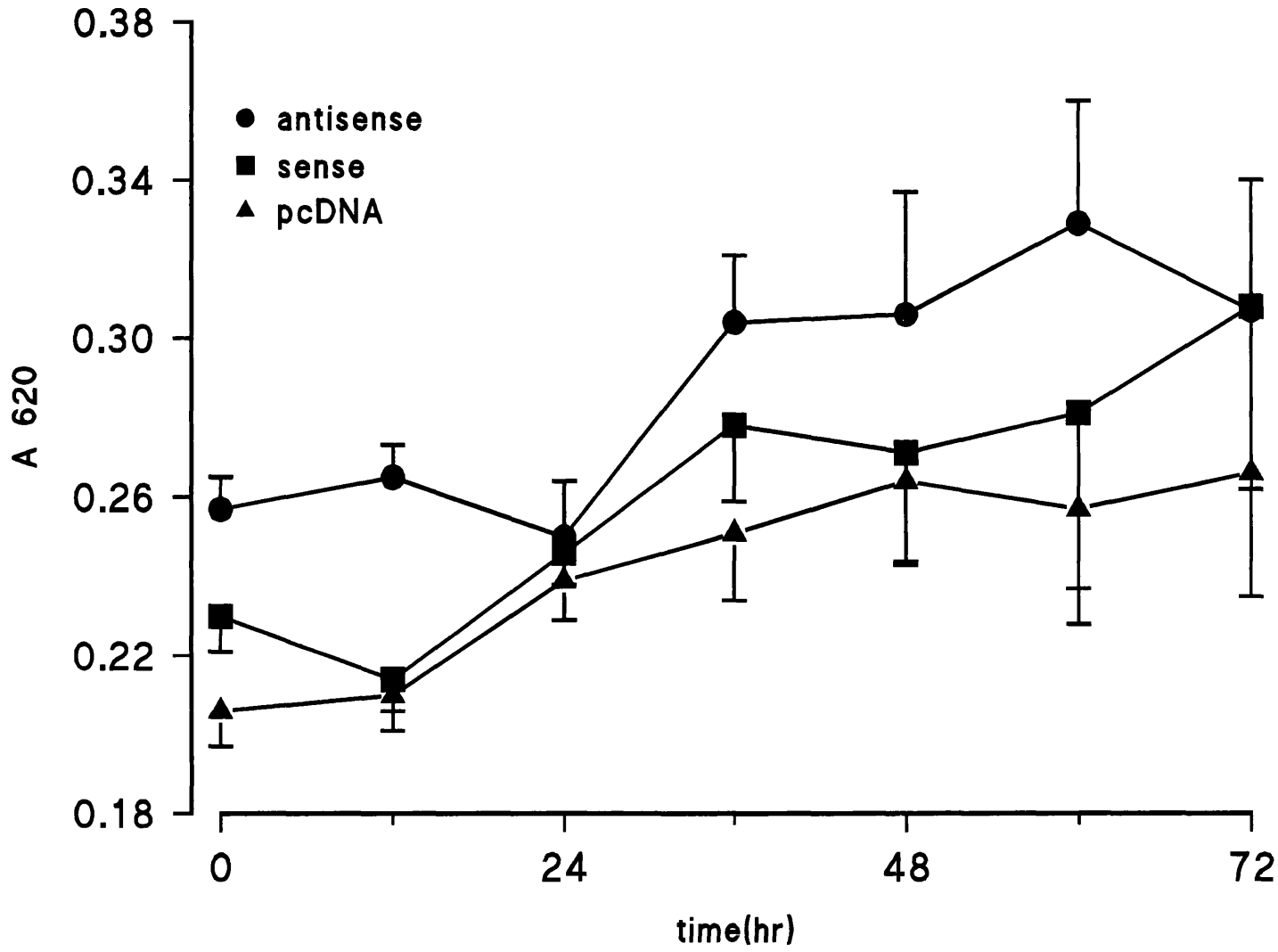
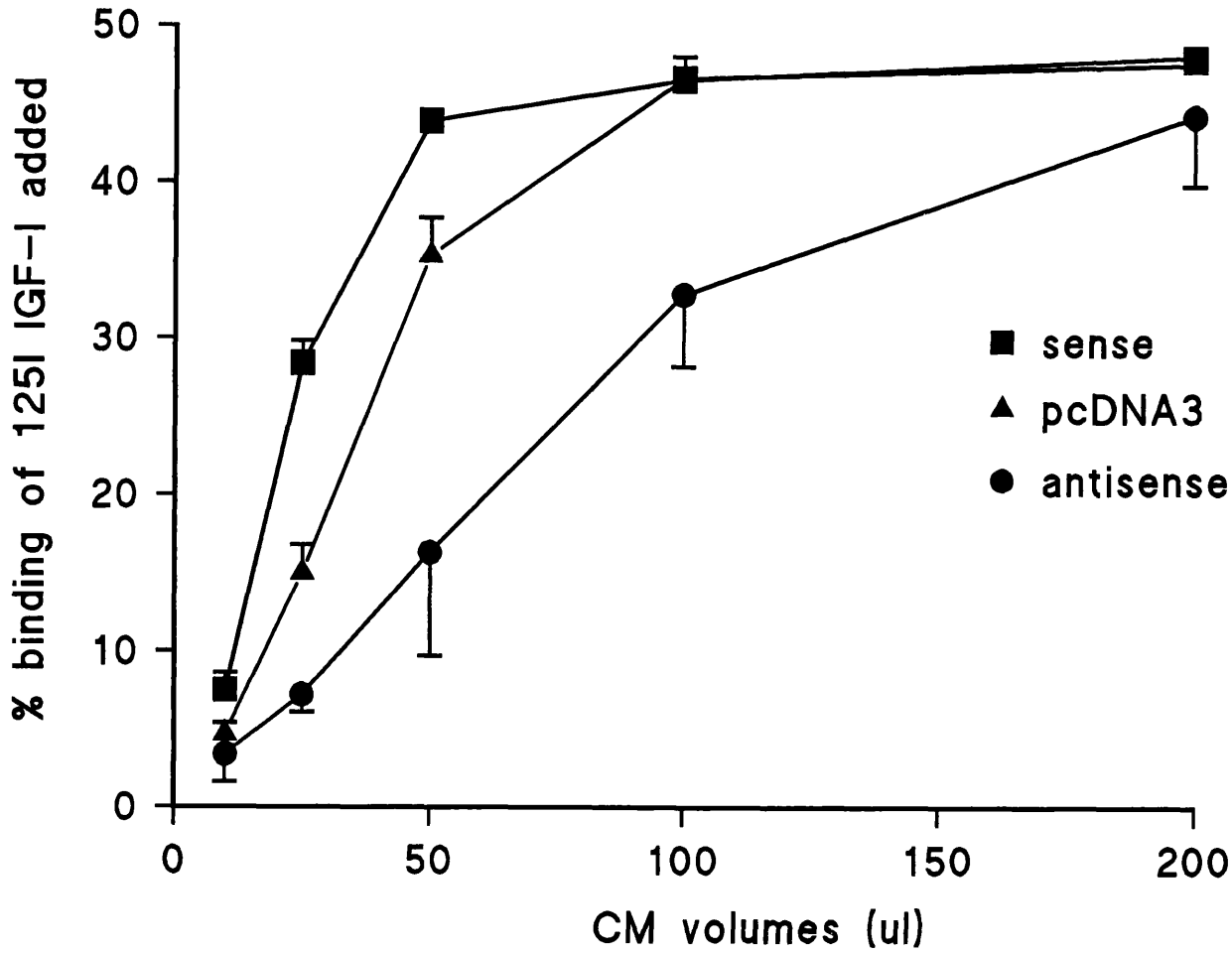


Fig 3.3.3.3 Charcoal binding assay on 24h CM from Clone 9 cells transfected with sense/antisense IGFBP-2 or pcDNA vector



CM from pcDNA3 or sense IGFBP-2 transfected cells. In addition, sense-transfected cells appeared to secrete approximately twice as much IGFBPs into conditioned medium when compared to pcDNA transfected cells.

3.3.3.4 DISCUSSION

IGF stimulation of growth has been reported in numerous cell types. In body tissues and many cultured cells, endogenous IGFBPs act as modulators of IGF mitogenic actions. The indirect effect exerted on cell growth by treatment with exogenous IGFBPs has also been extensively reported (Jones and Clemmons, 1995a). Based on this evidence, it has been postulated, and in some cases demonstrated that, both the overexpression or the inhibition of expression of specific IGFBP genes can affect cell proliferation. Accordingly, using this hypothesis we tested if, in Clone 9 cells (a cell line responsive to IGF mitogenic stimulation), the inhibition of endogenous IGFBP-2 expression influenced the rate of cell growth. Preliminary results from the time course experiment presented here (paragraph *3.3.3.2*), show that under basal conditions (in the absence of added IGFs), the transfection of Clone 9 cells with either sense/antisense IGFBP-2 or pcDNA3 vector alone did not differentially affect cell growth under our culture conditions. Many interpretations of this finding are possible, but it should be noted that this was the result of a single experiment. It is essential to confirm this result and to characterize in more detail the level of inhibition of expression before drawing any conclusions.

In cells that produce endogenous IGFs and that are responsive to the mitogenic effect of IGF, transfection with antisense IGFBP constructs has been reported to stimulate cell proliferation after a variable time and under different culture conditions. Corkins *et al.* (1995) showed a 35% increase in cell growth in a rat intestinal epithelial cell line (IEC-6) transfected with antisense IGFBP-2 after 3 days culture in 5% FBS. The same research group (Parks *et al.*, 1999) recently reported that human colon carcinoma (Caco-2) cells transfected with antisense IGFBP-3 displayed an increased growth rate, compared to pcDNA3-transfected cells, after only 8 days in culture (either in serum free or serum containing medium). On the other hand, the effect on cell growth of overexpression of sense IGFBP-2 has also been investigated. Human embryonic kidney fibroblasts stably transfected with IGFBP-2, displayed impaired cell growth after 5 days of culture, compared to control cells (Hoflich *et al.*, 1998). After 3 day of culture, rat glioma cells (C6) transfected with IGFBP-2 cDNA and expressing a high level of protein,

grew at the same rate as control cells, while identically transfected clones expressing low level of IGFBP-2 displayed impaired growth (Bradshaw *et al.*, 1999). A direct comparison of the effects of sense or antisense transfection may not be appropriate. In the antisense technique, the expression of the targeted gene is generally not completely abolished. In the cases above, with antisense IGFBP-2 and IGFBP-3, (Corkins *et al.*,1995; Parks *et al.*, 1999) a 54 and 55 % reduction of the corresponding mRNAs was observed, respectively. In the case of IEC6 cells transfected with the antisense IGFBP-2 construct, the concentration of IGFBP-2 in CM was reduced by 68% after 6-8 days of culture. A 60% reduction of IGFBP-3 RNA and 80% decrease in protein concentration was obtained by using antisense IGFBP-3 oligodeoxynucleotides (ODN) (Oh *et al.*, 1995). However the use of antisense oligonucleotides, is a different technique which transiently inhibits the expression of a target gene. As a consequence, freshly transfected cells are needed for every experiment and a wide variability in the inhibition of gene expression is commonly experienced. For this reason many researchers prefer to create cell lines stably transfected with antisense constructs. Of course, not even this technique is devoid of drawbacks. The transfected foreign gene is integrated randomly into the cellular genome and the location site of integration plays an essential role in the level of expression of the exogenous DNA. However, as most of the expression vectors carry a neomycin-resistance gene, transfected cells can be selected in the presence of a specific drug (usually G418), and a clonal population can be grown separately from a single resistant cell. After the assessment of the efficiency of the reduction of mRNA transcription and protein expression, the best clones can be chosen and expanded.

Our experiment *3.3.3.2* was carried out with a mixed population of stably transfected cells. Although this might not be an ideal situation because the overall inhibition of gene expression corresponds only to the average between highest and lowest antisense mRNA expressing cells, Corkins *et al.* (1995) successfully demonstrated that a non-clonal population of IEC cells transfected with antisense IGFBP-2 grew faster than control cells.

With regard to the efficiency of the inhibition of IGFBP-2 expression achieved in antisense-transfected Clone 9 cells, although encouraging, only a limited amount of information on the total IGF-binding capacity of the CM can be derived from the charcoal binding assay and more detailed analysis are necessary. Not only should the specific decrease of IGFBP-2 in the conditioned medium be verified by

Western blotting, but also any changes in general protein secretion or in the expression of other classes of IGFBPs should be carefully analysed. In fact, the overexpression or the inhibition of the expression of one specific IGF-binding protein can induce a compensatory cellular response resulting in an alteration of the IGFBP profile or even inducing IGF expression (Bradshaw *et al.*, 1999; Park, 1999).

In the case of the MTT experiments what may be required in order to see an effect, is the use of a clonal population of cells whose IGFBP-2 expression is greatly inhibited. However, while our study was progressing, most reports on the use of a sense or antisense strategy to highlight the role IGFBP-2 on cell proliferation supported an inhibitory effect for IGFBP-2 on cell proliferation (Corkins *et al.*, 1995; Hoflich *et al.*, 1998; Bradshaw *et al.*, 1999). These results are in line with the well documented general inhibitory effect of IGFBP-2 on IGF action and do not support its role as a positive regulator of cell growth. This latter hypothesis arose from early reports showing synergistic effects of IGFBP-2 on IGF metabolic or mitogenic action in different cell systems (Bar *et a.,* 1989; Bourner *et al.,* 1992) and it was corroborated by the finding that many tumours express high levels of IGFBP-2 (Elmlinger *et al.*, 1998, Mishra *et al.*, 1997; Cohen et al., 1993; Fuller *et al.*, 1999; Ho and Baxter, 1997b; Kanety *et al.*, 1993, Kanety *et al.*, 1996; Karasik *et al.*, 1994). Moreover, Slootweg *et al.* (1995) described an IGF-independent effect of IGFBP-2 in rat osteosarcoma cells. Hoflich *et al.* (1998) investigated if the high level of expression of IGFBP-2 in tumours could be indicative of its involvement in the pathogenesis or in the progression of tumour growth. In light of the growth inhibitory effect, which is obtained either by overexpressing IGFBP-2 in human fibroblasts, or by adding medium conditioned by these cells to colon carcinoma cells, the authors suggested an alternative interpretation. Rather than a synergistic effect on IGF-stimulated growth, the increased secretion of IGFBP-2 in the CM of cultured tumour cells should be seen as a cellular defensive reaction aiming to limit the high level of proliferation.

Of course, the lack of evidence of a positive role for IGFBP-2 in transfection studies is not sufficient to exclude the occurrence of this effect. For example, none of the systems used could really discriminate between the action of soluble or membrane-associated IGFBP-2. This is a difficult area to address. The higher potency shown by des(1-3)IGF-I compared to IGF-I on the stimulation of tyrosine phosphorylation of a 180kDa protein in wild type Clone 9 cell, could be exploited for the purpose. As mentioned above, in the tyrosine phosphorylation assay the conditioned medium is discarded and only the

monolayer adherent IGFBPs are present. We have hypothesised that these immobilized IGFBPs may be responsible for the impaired effects of IGF-I compared with its analogue des IGF-I. A cell system devoid of immobilized IGFBPs (antisense IGFBP-2 transfected Clone 9 cells) would be an appropriate model to test this hypothesis. If the difference between IGF and des IGF was not apparent, it would be additional proof of the inhibitory effect played by membrane-associated IGFBP-2 in wt Clone 9 cells. If, instead, the difference between IGF and des(1-3)IGF persists in the absence of IGFBP-2 expression, it may be due to the presence of an additional IGFBP associated to the monolayer (IGFBP-5 for example), or due to completely unrelated reasons. We have shown some limited evidence suggesting Clone 9 cells secrete IGFBP-5 (paragraph *3.3.1.b.3*), which is known to associate to the cell membrane and to ECM. In this respect, if the secretion of this binding protein by Clone 9 cells can be confirmed, the effect of alternative suppression of the IGFBP-2 or the IGFBP-5 gene may be helpful in elucidating the specific role played by each IGFBP in this cell model system.

4. General discussion

It is not the intention of this final chapter to repeat the discussion of the experimental results obtained in the current work. These are fully described at the end of each experimental section. Instead we intend to conduct a broader discussion of two particular areas of the current work, namely a comparison of the IGF axis we have described for Clone 9 cells and that which exists in primary cultures of hepatocytes, and also some further considerations as to the use of antisense technology in relation to the experimental systems.

The work undertaken in this study has provided a large range of useful information on various biological aspects of IGFBP-2, and at the same time it has lead to an enrichment of our knowledge in different research areas. We started from DNA cloning and mutagenesis and then, introducing for the first time the use of the baculovirus expression system in our Institute, we expressed wt and mutant IGFBP-2 proteins and we compared their affinity for IGF. During this first part of the project we also produced and purified wt IGFBP-2 (and its PGD mutant) which we utilised in the second part of our research aiming to investigate IGFBP-2 association with the plasma membrane of cultured cells. In this area we tried many different approaches and we finally succeeded in demonstrating the association of endogenous IGFBP-2 to cell membranes of Clone 9 cells, an adult rat liver cell line, that secrete only IGFBP-2 into CM. We then moved to the last part of our project, investigating the role of endogenous IGFBP-2 in modulating the IGF-IGF-R axis. We established two bioassays which helped to enlighten different aspects of IGFBP-2 modulation of Clone 9 cell responsiveness to IGFs. In our acute, cell signalling-type bioassay, IGF action is probably influenced by monolayer-associated IGFBPs, while in our long-term cell proliferation assay, both soluble and monolayer-associated IGFBP-2 can affect growth factor activity. We observed that in the Clone 9 cell line, des(1-3)IGF-I, an IGF-I analogue with normal affinity for IGF-IR and decreased affinity for IGFBPs, appeared more potent than IGF-I in stimulating both protein phosphorylation and cell proliferation. These results led us to hypothesise that the negative modulation of IGF-I action could be due to the presence of endogenous IGFBP-2, regardless of its soluble or monolayer association. According to

this hypothesis, in the absence of the endogenous binding protein, the activity of IGF-I and des(1-3)IGF-I would be expected to be similar. We worked to create a cellular model suitable to test this hypothesis, by transfecting Clone 9 cells with an antisense IGFBP-2 construct. At this time, only preliminary results have been obtained from our antisense IGFBP-2 transfected Clone 9 cell model. Further investigation is required to establish the level of inhibition of IGFBP-2 gene expression achieved and the effect that this inhibition has on cell growth in our Clone 9 cell model.

The Clone 9 cell line was derived from normal adult rat liver (Weinstein *et al.*, 1975). The cells display an epithelial phenotype with a cuboidal or polygonal cell shape, and grow tightly packed, but not overlapping, in discrete colonies which eventually become confluent. When the cell line was originally established, selection of these cells from liver primary culture was facilitated i) by low cell density plating, which enables the detection of epithelial clusters over the widely spreading fibroblasts, and ii) by using a medium with a low percentage of FBS (3-5%), which favours growth of epithelial over fibroblastic cells. Weinstein and coworkers excluded the possibility that Clone 9 cells underwent tumour transformation, because no viral particles were detected in the cell cytoplasm and because cells were unable to grow in soft agar. However, Clone 9 cells lacked hydrocortisone-inducible tyrosine aminotransferase, which is typical of hepatocytes and which is also retained in hepatoma cells (Weinstein *et al.*, 1975). Therefore the authors concluded that Clone 9 cells were likely to be derived from hepatic parenchyma cells although an origin from bile duct epithelial cells could not be excluded. As Clone 9 cells are often referred to as "hepatocytes" (Matovcik *et al.*, 1990; Brown *et al.*, 1986) and as the cells, which are commercially available, displayed the expected phenotype, we would have probably continued to consider them as hepatocytes if some of our results had not suggested otherwise.

The IGFBP secretion profile of the different liver cell types has been characterised in detail by Scharf *et al.* (1995). Adult rat hepatocytes secrete IGFBP-1, 2 and 4 (in decreasing order of abundance), and do not produce IGFBP-3. Fat-storing cells secrete IGFBP-2 and 4, while endothelial cells and Kupffer cells produce IGFBP-2 and 3. The authors suggest that this compartmentalization of IGFBP secretion might have the function of preventing intracellular formation of 150kDa ternary complex in the hepatocytes which secrete both ALS subunit and IGF-I, but not IGFBP-3. Also Schmid *et al.* (1992)

reported that adult rat hepatocytes secrete a major 32kDa IGFBP and a lesser amount of IGFBP-2. In contrast to these reports, other authors claim that IGFBP-1 is undetectable in normal adult rat liver, and its secretion is only apparent in regenerating liver (Mohn *et al.*, 1991). Foetal rat hepatocytes secrete IGFBP-1, -2 and -4 similarly to what was reported by Scharf in adult rat hepatocytes (Menuelle *et al.*, 1995).

In Clone 9 cell conditioned medium only one band of approximately 32-34kDa was detectable by IGF-I ligand blotting. In agreement to what previously shown by Yang *et al.* (1990), we identified this protein as IGFBP-2, as it reacted with an anti IGFBP-2 (but not by anti IGFBP-1 antibody), in Western blotting (data not shown). Conversely, on Clone 9 cell membranes, in addition to IGFBP-2, another IGFBP of slightly smaller Mr was revealed and our immune precipitation data suggest this protein might be IGFBP-5. The difference between IGFBP profile in Clone 9 cells (IGFBP-2) and that described in the literature for hepatocytes (IGFBP-1>IGFBP-2>IGFBP-4) might reflect an adaptation of the cell line to culture conditions and does not exclude the possibility that the two cell types have the same histological origin. In this respect it should be noted that IGFBP secretion in hepatocytes varies under different conditions, such as foetal life (Menuelle *et al.*, 1995), liver regeneration (Mohn *et al.*, 1991), and various tumour states (hepatoma cell lines, Yang *et al.*,1990 and Scharf *et al.*,1998).

We then compared the IGF-IGF-R axis of hepatocytes and Clone 9 cells. Primary cultures of human hepatocytes, express IGF-I but not IGF-IR. Conversely, they express IGF-IIR, but not IGF-II (Scharf *et al.*, 1998). The expression of IGF-I and the lack of the expression of IGF-IR receptor, though characteristic of adult human and rat hepatocytes (Schmid *et al.*, 1992; Scharf *et al.*, 1998), cannot be adopted as a marker for hepatocytes as it is a reversible condition. For instance, IGF-IR is expressed in foetal hepatocytes, in regenerating liver (after hepatectomy) and in some hepatoma cells (Caro *et al.*, 1988). Furthermore, PLC cells (human hepatoma cells) express both IGF-I and IGF-II receptors and none of the IGF peptides (Scharf *et al.*, 1998). Our data show that Clone 9 cells express IGF-I and IGF-IR and, although we did not obtain definitive evidence of the IGF-IIR, our results are not inconsistent with its presence, which has previously been described in this cell line (Matovcik *et al.*, 1990 and Brown *et al.*, 1986).

Several studies have been published which aimed to identify the receptor responsible for mediating IGF action in hepatocytes, but results were not always directly comparable, due to the different

experimental conditions utilised. Scharf *et al.* (1996) reported that in adult rat hepatocytes insulin and IGF-I decreased IGFBP-1 and IGFBP-2 secretion, but they upregulated IGFBP-4. Based on the lower potency displayed by IGF-I in comparison to insulin, the authors postulated that the insulin R mediated the action of both hormones. Raper *et al.* (1995) showed that both IGF-I and IGF-II (at a concentration of 2nM) stimulated ^{3}[H]thymidine uptake in adult rat hepatocytes, but the two peptides acted through different mechanisms. IGF-I elevated cellular cAMP levels and its action was decreased by somatostatin (SS14) and by a specific antagonist of the adenyl cyclase signal transduction pathway (2'-5' dideoxyadenosine, DDA). Conversely, IGF-II-stimulated thymidine uptake was not accompanied by an increase of cAMP and was unaffected by the addition of these two compounds. In addition, ^{125}I IGF-II binding to the hepatocytes was specific and it was likely to be mediated by IGF-IIR (as it was competed by IGF-II, but not by IGF-I or insulin). ^{125} I insulin also bound specifically to its receptor and it was significantly displaced by IGF-I, but not by IGF-II. The authors concluded that, IGF-IR expression being very low in adult liver hepatocytes, the IGF-I-stimulated increase in cAMP and thymidine uptake might be partially mediated through the insulin receptor, while IGF-II, which did not bind efficiently to the insulin receptor, might act through IGF-IIR. Also Kimura and Ogihara (1998) reported that IGF-I and IGF-II stimulate DNA synthesis and cell proliferation through two different mechanisms in primary culture of adult rat hepatocytes. IGF-I induced DNA synthesis was increased by cAMP-elevating agents, by an α_1-adrenoceptor agonist and by phorbol myristate acetate (PMA). IGF-I signalling was mediated by tyrosine kinase, phospholipase C, PI(3)K and p70 S6K. In contrast, IGF-II stimulated DNA synthesis was inhibited by cAMP-elevating agents, by an α_1-adrenoceptor agonist and by PMA. IGF-II signalling was mediated by Gi protein, MAP kinase and p70 S6K. IGF-II was 100 fold more potent than IGF-I in inducing DNA synthesis and, while IGF-I-stimulated cell proliferation was inhibited by high cell plating density, IGF-II action was unaffected. Moreover, although in this paper the nature of the receptors mediating IGF effects was not specifically addressed, the authors reported that IGF-I-induced thymidine uptake in adult rat hepatocytes was inhibited by the addition of an anti IGF-IR antibody , and IGF-II action was antagonised by an anti IGF-IIR, but not vice versa.

Despite the suggestions of Raper (1995) and Kimura and Oghiara (1998) of an involvement of IGF-IIR as a mediator of IGF-II signalling, a signal transduction pathway for the IGF-IIR has not been

convincingly demonstrated. Both IGF-I and IGF-II actions are believed to be mediated principally through the IGF-IR. An alternative mediator of the biological effects of IGF-II in foetal rat hepatocytes has been suggested by Menuelle *et al.*(1995). The authors propose that in these cells, both IGF-II and insulin-stimulated glycogen synthesis is mediated by the insulin receptor. More recently, the same group of researchers has expanded this study, showing that in the same cell type, insulin and IGF-II also induced DNA synthesis. In this case, insulin appeared to act through its own receptor, while IGF-II was more likely to act through the IGF-IR (foetal rat hepatocytes express IGF-IR) (Menuelle *et al.*, 1999). However in this case, the attempt to discriminate between signals occurring through the insulin or the IGF-I receptor might appear slightly artificial, as when both receptors are expressed in a cell type, they may form hybrid receptors (Siddle et al., 1999). The hypothesis of IGF-II signalling through the insulin receptor finds strong support in the study of Morrione *et al.*, 1997, who demonstrated IGF-II ability to stimulate cell proliferation in R-/IR fibroblastic cells. These cells were originally derived from *igf1r* nullizygotes (thus lacking IGF-IR) and have been transfected to obtain overexpression of the insulin receptor. In this model, both insulin and IGF-II (at 50ng/ml) stimulated cell proliferation, DNA synthesis, insulin receptor autophosphorylation and phosphorylation of IRS-1, the major protein substrate of the insulin receptor. In contrast, IGF-I, even at concentrations as high as 200ng/ml, failed to stimulate R-/IR cell proliferation. The authors suggest that the observed difference in functionality between the two IGFs can be explained by considering their relative affinity for the insulin receptor. IGF-II affinity for the insulin receptor is only 1/10 of that of insulin, whereas IGF-I binding to the insulin receptor is 50-100 times lower than insulin (DeMeyts *et al.*, 1994).

In Clone 9 cells, IGF-I was generally more effective than IGF-II or insulin in stimulating either proliferation or protein phosphorylation. ^{125}I IGF-I or ^{125}I IGF-II affinity labelling of Clone 9 membranes revealed the presence of the 130kDa α subunit of IGF-IR. We believe that IGF-IR receptor is the main mediator of IGF-I and IGF-II biological actions in Clone 9 cells. However, the expression of insulin receptor on Clone 9 cells was not specifically investigated and in the light of recent findings (Morrione *et al.*, 1997; Menuelle *et al.*, 1999) the involvement of insulin receptor in the mediation of insulin and IGF-II effects on Clone 9 cells cannot be excluded.

In summary, Clone 9 cells have been found to be a suitable cellular model for studying some of the IGFBP-2 biological functions of IGFBP-2 as this cell line expresses all components of the IGF-IGFR-IGFBP axis, and especially cell membrane associated IGFBP-2. However, whether Clone 9 cells can be considered a physiological model for adult rat hepatocytes is another question and this would require a specific histological characterisation of these cells.

Our choice of studying IGFBP-2 function by following a "lack of a function" (gene expression inhibition by antisense mRNA), was dictated by our main aim to investigate the role played by the IGFBP-2 fraction which is associated to the cell membranes. The IGF inhibitory effect of soluble IGFBP-2 is already well established. To investigate the role of cell membrane associated IGFBP-2 we could either increase or decrease the amount of this IGFBP bound to the cell surface. As we were unable to detect cell surface binding of exogenous IGFBP-2, the utilisation of methods based on the addition of the binding protein to cell monolayers were precluded. Thus, we decided to block endogenous IGFBP-2 production by Clone 9 cells by transfecting them with an antisense IGFBP-2 construct. We attempted to deduce some useful information on the role of endogenous (soluble and monolayer-associated) IGFBP-2 by comparing cell growth of wt Clone 9 cells or antisense transfected, either following IGF treatment or under basal conditions. In the latter case, the endogenous production of IGF-I might act in an autocrine fashion to sustain Clone 9 cell proliferation, and therefore its modulation by endogenous IGFBP-2 might be of physiological significance. Moreover, it might be possible that, in the absence of endogenous IGFBP-2 (in antisense transfected cells), the binding of exogenously added protein is more easily demonstrated. In this case it could be possible to treat antisense transfected Clone 9 cell monolayers with IGFBP-2 and study the specific role of the monolayer-associated protein on cell growth avoiding the interference of the soluble IGFBP-2. On the other hand, we planned to elucidate the specific role of monolayer-associated IGFBP-2 by comparing wt and antisense transfected Clone 9 cells in IGF-stimulated protein phosphorylation assays (where, under our experimental conditions, only monolayer associated IGFBPs are present). Of course all these future experiments are based on the assumption that the transfection of Clone 9 cells with antisense IGFBP-2 drastically suppresses the expression of the endogenous binding protein.

Although we describe the use of full length antisense IGFBP-2 stably transfected into Clone 9 cells via a pcDNA3 expression vector, from a practical point of view, due to the progress made in

antisense technology and the knowledge achieved in this field, there is a large range of options available to maximise the efficiency of inhibition of gene expression. For example, a choice could be made on whether pre-mRNA rather than mature mRNA should be targeted. It has been proposed that the subnuclear localization in which the antisense molecule interacts with its target can affect the efficiency of expression inhibition. Chuang (1992) suggests that targeting antisense oligos to spliceosomes could prevent intracellular degradation and increase the chances of meeting the target sequence. Alternatively, inhibition of gene transcription could be achieved by directing triple helix-forming-oligonucleotides to the gene promoter region. IGFBP-2 promoter has been studied in mouse (Landwehr *et al.*, 1993), rat (Brown and Rechler, 1990), human (Binkert *et al.*, 1992), pig (Song *et al.*, 1993) and chicken (Schoen *et al.*, 1995). It lacks TATA or CAAT motifs, but in the proximal region upstream of the 5'end of the gene several G/C boxes have been identified and shown to mediate basal IGFBP-2 promoter activity (Boisclair *et al.*, 1993; Kutoh *et al.*, 1993). Recently, other more upstream sequences able to augment basal IGFBP-2 promoter activity have been identified in liver derived cells and even the presence of a gene transcription silencer has been demonstrated (Badinga *et al.*, 1998; Kutoh *et al.*, 1999). The latter acts as a negative modulator of IGFBP-2 expression in proliferating BRL-3A cells (a rat liver cell line) cultured at low density. In the same cell line, IGFBP-2 expression increases when confluence is reached and cells are arrested at the border between G1/S phase possibly by activating an enhancer element which counteracts the silencer function. Nuclear factors interacting with the silencer and enhancer DNA regions have been identified and their role as a repressor and an activator has been suggested. In the light of these findings other methods of blocking IGFBP-2 expression could be considered. If the identification of the repressor is achieved, it might be possible to increase the intranuclear concentration of this factor and possibly potentiate its transcription silencing activity. An alternative strategy may be to transfect the cells with double stranded DNA "decoys", which, reproducing transcription factor binding site sequences, might sequester these factors and inhibit transcription. A more definitive solution would be to employ cells derived from IGFBP-2 knock out mice. These were established by Wood et al., (1993) and were shown not to display any obvious change in phenotype. However, the authors have suggested that the lack of IGFBP-2 might be masked by a compensative effect from the other class of IGFBPs. This possibility should be also taken into account if such a model was chosen to test IGFBP-2 functions.

Considerations of this type are important in the current work, as initial observations from populations of Clone 9 cells stably transfected with antisense IGFBP-2 indicate that general protein secretion by these cells is decreased questioning the specificity achieved by the full length antisense BP-2 construct. Experiments in this area are continuing in our laboratory.

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Appendix: papers published in support of the thesis

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Growth hormone stimulates the secretion of insulin-like growth factor binding protein-2 (IGFBP-2) by monolayer cultures of sheep costal growth plate chondrocytes

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Abstract

Using monolayer cultures of costal chondrocytes established from four week old Clun Forest lambs, we have demonstrated that, under serum free conditions the cells release three IGFBPs (32, 29 and 21 kDa) into the medium. The most abundant of these – the 32 kDa BP – was shown to be IGFBP-2 by Western blotting. Furthermore we demonstrate that the levels of IGFBP-2 in conditioned medium are acutely increased (6, 12 and 24 h time points) following treatment of cells with bovine GH (1–100 ng/ml).

In a parallel set of experiments, using ovine fibroblasts (derived from dermis) we show that IGFBPs of Mr 32, 29 and 21 kDa are also secreted by this cell type. However the relative abundance of these BPs differed from that seen in the chondrocyte cultures, with the 21 kDa species now the most abundant. In addition, prolonged exposure of autoradiographs indicated that fibroblasts secreted a higher Mr IGFBP (most probably ovine BP-3) that was not detected in any of our chondrocyte cultures. Most significant however was the demonstration that bGH did not dramatically affect the levels of IGFBPs in fibroblast cell cultures. We conclude that GH stimulates BP-2 production from chondrocytes and this is a cell-type specific effect in as much as it is not replicated in cultures of dermal fibroblasts. (Mol Cell Biochem **162**: 145–151, 1996)

Key words: ovine chondrocytes, growth hormone, IGFBPs

Abbreviations: BCIP – 5-bromo-4-chloro-3-indolyl phosphate; CM – conditioned medium; DMEM – Dulbecco's Modified Eagles Medium; FCS – foetal calf serum; IGF – insulin-like growth factor; IGFBP – insulin-like growth factor binding protein; HBSS – Hanks' Balanced Salt Solution; GH – growth hormone; NBT – nitroblue tetrazolium; SFM – serum free medium; TBS – tris buffered saline

Introduction

The insulin-like growth factors (IGF-I and IGF-II) are small (7.5 kDa) polypeptides which display mitogenic, differentiating and metabolic activities (reviewed in ref [1]). *In vitro* and *in vivo* these actions are modulated by non-covalent association with well-characterised IGF binding proteins (IGFBPs), six of which have been cloned for both rat and human [2]. Association of IGFs with their binding proteins has been reported to enhance [3] or inhibit [4] their activities.

One of the most important functions of IGF-I, in association with pituitary growth hormone (GH), is the regulation of longitudinal skeletal growth in mammals, a process which is principally achieved by the differentiation, clonal expan-

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sion and subsequent ossification of chondrocyte populations within the epiphyseal growth plates [5–7]. Although the exact role of GH and IGF-I in this process is still not clearly defined, it is evident that in the case of the latter hormone its activity at the growth plate will be influenced by the presence, locally of the IGFBPs discussed above. Very little data is available on IGFBP production by growth plate chondrocytes and even less on the hormonal regulation of secretion of these proteins from this cell type. Because of this we have investigated the secretion of IGFBPs from differentiated, monolayer cultures of ovine costal chondrocytes. We report that under our serum-free culture conditions, chondrocytes release principally IGFBP-2 (~32 kDa) with lesser amounts of a 29 and 21 kDa IGFBP. Further, we demonstrate that in this culture system bovine GH stimulates (2-fold increase) the secretion of IGFBP-2. We discuss our results in the context of a possible interplay between GH and IGF-I in the regulation of growth plate metabolism.

Materials and methods

For tissue culture, DMEM, Ham's F12 and foetal calf serum were from Gibco BRL (Inchinnan, Scotland). Penicillin/streptomycin, Hanks' balanced salt solution and trypsin-EDTA were from ICN Biomedicals (Bucks, England). Collagenase was from Boehringer (Sussex, England), DNase from Sigma (Dorset, England) and donor calf serum from Tissue Culture Services (TCS - Bucks, England). Tissue culture plasticware was from CoStar UK (Essex, England); Alcian Blue, protamine sulphate, activated charcoal, goat anti-rabbit IgG alkaline phosphatase conjugate and reagents for electrophoresis were from Sigma. Rabbit antibovine IGFBP-2 was from TCS. This is a polyclonal rabbit antibody raised to bovine IGFBP-2 purified from the MDBK cell line. It cross-reacts with ovine BP-2 and shows < 0.5 % cross-reactivity with IGFBPs 1, 3, 4 and 5 [8]. Recombinant human (rh) IGF-I was from Bachem UK (Essex, England) and recombinant bovine growth hormone (rbGH-Met-1 . . . Leu 127, lot no M-118-08308) was a gift from Dr R Collier, Monsanto Corp, St Louis, MO, USA. Electrophoresis apparatus Protean IIxi was from Bio-Rad (Herts, England) and blotter model TE42 was from Hoeffer Scientific Instruments (San Francisco, USA).

Cell culture

Costal chondrocytes were prepared from the growth plates of 4 week old male lambs. After removal of the rib cage each individual rib together with the joint above the growth plate was separated and scraped free of all tissue using a scalpel. The growth plates (12 per animal) are about 1–2 mm thick, milky in colour and easy to separate from the adjoining bone. They were removed sterilely and washed twice in Petri dishes with 10 ml DMEM/Ham's F12 media with no serum. The growth plates were pooled and chopped into 1 mm sq pieces using a scalpel and then added to a sterile enzyme solution – 0.12% collagenase, 0.02% DNAse in 20 ml DMEM/Ham's F12 with 10% donor calf serum. This digestion was left at 37°C for 18 h after which time most of the chondrocytes were isolated from the cartilage. Cells were washed × 3 seeded into 75 cm^2 flasks and frozen after 7 days in culture and one passage. For experimental purposes costal chondrocytes were seeded into 12-well tissue culture plates at 10^5 cells/well in complete medium (DMEM/10% FCS/100 units ml^{-1} penicillin/streptomycin). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2, for either 7 or 14 days. Prior to the experiments, cells were washed (× 2 in Hank's Buffered Salt Solution HBSS) and incubated with 500 ul fresh SFM ± bovine GH (bGH – final concentration 0–100 ng/ml). Incubation was continued for 6–72 h, following which medium was removed, centrifuged briefly to remove cell debris and stored at –20°C.

In order to confirm maintenance of chondrocyte phenotype in monolayer culture, cells were stained for the presence of proteoglycan using Alcian Blue according to Lindhal *et al.* [7]. To investigate the specificity of the effects of GH on chondrocyte cultures, replicate experiments were performed on cultures of sheep dermal fibroblasts. These were established from explants of skin removed from the inguinal region. Explants (approximately 1 mm sq pieces) were incubated in DMEM/10% FCS/100 units ml^{-1} Pen Strep in 10 cm tissue culture dishes. After 5–6 days in culture, fibroblasts were observed growing at the borders of explants. At this stage explants were picked from the culture with a scalpel and fibroblasts were allowed to grow to confluency. Cells were passaged with trypsin-EDTA and maintained in DMEM 10% FCS 100 U ml^{-1} Pen Strep. Cells of various passage were stored in liquid N_2 using DMEM:FCS:DMSO (50:40:10) as cryo preservant. For experimental purposes, fibroblasts were used between passage 2–5 and chondrocytes at passage 2 or 3. No differences in IGFBP secretion were evident in different passages. The hormonal treatment, incubation conditions and medium collection procedures were identical for chondrocyte and fibroblast cultures.

Western ligand blotting

This was performed according to the method originally described by Hossenlopp *et al.* [9]. 80 ul of conditioned medium (CM) was incubated with 20 ul × 5 strength non-reducing sample buffer (312.5 mM Tris.Cl pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 50 ug/ml bromophenol blue) at 100°C

for 3 min. Electrophoresis was for 4–5 h at 60 mA on a 12% polyacrylamide gel in a running buffer comprising 25 mM Tris.Cl (pH 8.3), 192 mM glycine, 0.1% (w/v) SDS. Gels were blotted at 130 mA for approximately 16 h (at 4°C) in 15 mM Tris (pH 8.3) 120 mM glycine, 5% (v/v) methanol. Following transfer, blots (16 × 14 cm) were incubated in Tris-Saline (TS – 150 mM NaCl, 10 mM Tris.Cl (pH 7.4)) containing 3% (v/v) NP-40 for 30 min, TS – 1% BSA for 2 h and TS – 0.1% Tween for 10 min. Blots were hybridised in 2 ml TS – 1% BSA 0.1% Tween containing 2 × 10^6 cpm ^{125}I-IGF-I for 16 h at 4°C. After washing – 2 × 15 min in TS – 0.1% Tween and 3 × 15 min in TS, autoradiographs were obtained by exposure to Fuji x-ray film in a cassette containing Dupont Cronex Li-Plus intensifying screens.

Immunoblotting

CM was processed exactly as described for ligand blotting with the exception that medium was prepared in reducing sample buffer containing 5% (v/v) 2-mercaptoethanol – final concentration. Following electrophoresis and transfer to nitro-cellulose, membranes were blocked by incubation for 30 min at room temperature in TS containing 2% (w/v) Marvel. Rabbit anti-bovine IGFBP-2 was diluted 1:2000 in TS – 0.2% Marvel and incubated for 2 h. After 4 × 10 min washes in TS, second antibody (goat anti-rabbit IgG alkaline phosphatase conjugate) diluted 1:1000 in TS – 0.2% Marvel was added and incubation was continued for a further 2 h. Blots were again washed for 4 × 15 min in TS and colour development was brought about by incubation in 0.1 M Tris.Cl (pH 9.5) containing 0.1 M NaCl 5 mM $MgCl_2$, .066% (w/v) NBT and .033% (w/v) BCIP. BCIP and NBT were added from 50 mg/ml stock solutions in 100% and 70% dimethylformamide respectively. Colour development was monitored over a 30–60 min period and the reaction was terminated by washing blots in TS.

Iodination

5 ug of IGF-1 was iodinated to a specific activity of 50–100 uCi/ug using the Iodogen coated tube method [10]. Labelled peptide was separated from free ^{125}I by gel filtration over a Sephadex G-10 column.

Solution phase assay of IGF binding proteins

This was performed as described originally by Conover *et al.* [11] with slight modifications. Briefly, varying volumes of chondrocyte conditioned medium (10–100 ul) were added to 100 ul of 50 mM Tris.Cl (pH 7.4), 0.5% BSA (assay buffer) containing ^{125}I-IGF-1 (25,000 cpm per tube). Total volume was made up to 300 ul with unconditioned medium. Binding of tracer was allowed to proceed overnight at 4°C. Following incubation, assay tubes were placed on ice and 600 ul of a solution containing 1% activated charcoal (Sigma Product No C-5385), 0.2 mg/ml protamine sulphate in assay buffer was added. After a further 10 min incubation on ice, charcoal was pelleted by centrifugation at 3000 rpm for 10 min. The supernatant, which contains the ^{125}I-IGF-I - IGFBP complexes was decanted. Charcoal pellets were counted and bound ^{125}I-IGF-I calculated by subtracting cpm in pellets from added ^{125}I-IGF-I counts.

Statistics

Data on binding of ^{125}I-IGF-I to chondrocyte monolayer CM in solution phase are presented as mean ± S.D. (n = 3), where n is the number of culture wells treated with the indicated concentration of bGH. Differences between untreated and GH-treated cultures were analysed by Student's *t*-test.

Results

Figure 1A indicates the results of a ligand blot of CM from monolayers of sheep growth plate chondrocytes stimulated with varying concentrations (0–100 ng/ml) of bGH for 6, 12 or 24 h (for experimental details see Materials and methods section). Under our culture conditions, these cells secrete 3 main IGFBPs at Mr 32, 29 and 21 kDa. It is also apparent that, for the most abundant 32 kDa IGFBP, GH has a stimulatory effect. Although it appears in Fig. 1A that the levels of the 29 and 21 kDa IGFBPs appear to be regulated in line with the 32 kDa protein, this was not a consistent observation. The identity of this 32 kDa IGFBP was established as IGFBP-2 after blotting with rabbit anti-bovine BP-2 (Fig. 1B). In addition, it is clear that the levels of BP-2 determined by immunoblot vary in parallel with those seen in the ligand blot. It should be noted that the ligand- and immunoblot represent gels containing different samples (from the same well) and is not a single nitrocellulose blot probed sequentially with ^{125}I-IGF-I and anti-BP-2 antibodies. It is also clear from Fig. 1B that neither the 29 kDa nor the 21 kDa IGFBP react with the anti-BP-2 antiserum suggesting that either these are not fragments of the 32 kDa BP-2 or, if they are, that the epitope(s) recognised by this antiserum have been lost. At 32 kDa, our observation of the Mr for the ovine BP-2 agrees well with 30.9 kDa calculated from the published sequence of this BP [12]. Attempts to identify the 29 and 21 kDa bands by immunoblotting were unsuccessful. Neither of these proteins reacted with polyclonal antiserum to human BP- 1, BP-4 or

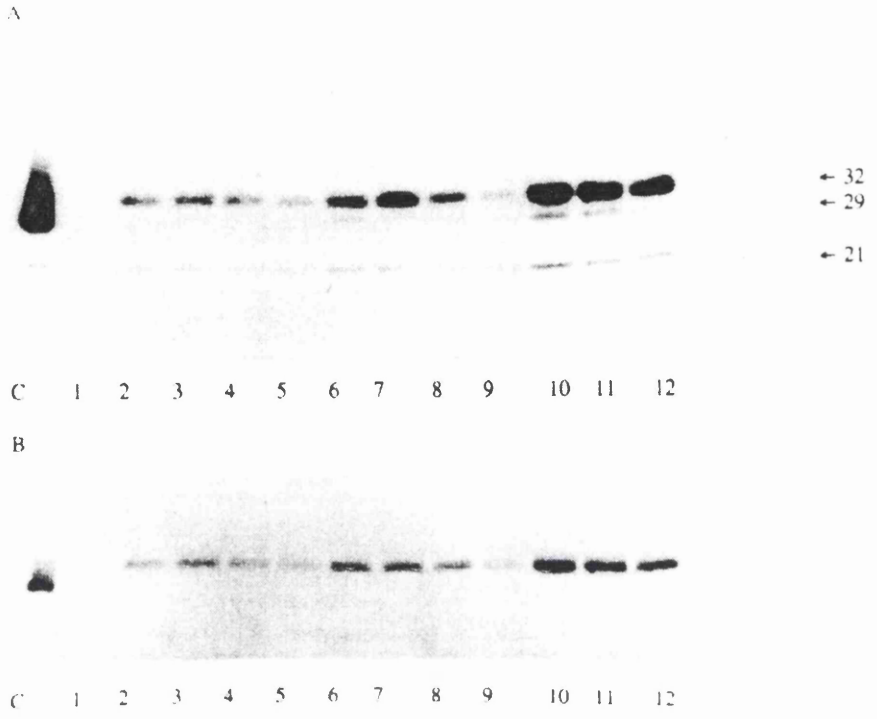

Fig. 1. Ligand blot (A) and immunoblot (B) of conditioned medium from chondrocyte monolayer cultures treated with varying amounts of bGH for either 6 h (lanes 1–4), 12 h (lanes 5–8) or 24 h (lanes 9–12). Concentrations of bGH are 0 (lanes 1, 5 and 9); 1 ng/ml (lanes 2, 6 and 10); 10 ng/ml (lanes 3, 7 and 11) and 100 ng/ml (lanes 4, 8 and 12). Lane C contains 10 ul of medium conditioned by the rat liver tumour cell line – Clone 9 which secretes the 30 kDa IGFBP-2 exclusively [24]. Note that ruminant BP-2 at 32 kDa has a higher Mr than the rat protein. For the immunoblot, key to the legend is as for ligand blot. The blots shown are representative of duplicate blots carried out on two separate experiments. 80 ul of conditioned medium were analysed by both ligand- and immunoblot as described in Materials and methods.

BP-5. (Antiserum to BP-6 was unavailable to us at this time). They are also unlikely to represent fragments of BP-3. In respect of the 29 kDa BP, it should be noted that on prolonged exposure of the autoradiograph, this band frequently presented as a doublet (data not shown). Stimulation of the IGFBP levels at the 6, 12 and 24 h time points was mirrored by our solution phase IGFBP data (Fig. 2). At 1 and 10 ng/ml bGH for each of the 6, 12 and 24 h time points, $p < 0.01$ (compared to IGFBP levels in control wells). At 100 ng/ml bGH $p < 0.05$ compared to corresponding controls for each of the time points. This fact, together with the level of ^{125}I-IGF-I binding by CM suggested to us that little IGF-I/IGF-II was released by these cells. This was confirmed following assay of acid gel filtration fractions from CM. We would emphasise that this stimulation of IGFBP secretion by GH is not simply a result of an increase in cell number following hormone treatment. At the range of GH concentrations used in the present study no increase in cell number was seen (data not shown).

A major concern during the monolayer culture of chondrocytes is the well-reported dedifferentiation of the cells that occurs on serial passage [13]. Although we always worked with passage 2 or 3 cells, we were careful to confirm chondrocytic phenotype by staining for proteoglycan with Alcian Blue. Despite this and in order to confirm the specificity of the effect of GH on monolayer cultures of chondrocytes, we conducted parallel experiments with sheep dermal fibroblasts. We present the results of these experiments in Fig. 3A and this data should be compared with that presented in Fig. 1A. It is immediately obvious that there are differences in the nature and relative intensities of the various IGFBP species. For example the ratio of 21 kDa to 29 or 32 kDa protein is much higher in the fibroblast conditioned medium than in the chondrocyte conditioned medium. In addition there appears to be very little consistent effect of bGH on the levels of any of the IGFBP species in fibroblast cultures. In order to confirm these differences in expression of IGFBPs between fibroblasts and chondrocytes we over-exposed an autoradiograph of a ligand blot with CM from both fibroblast and chondrocyte CM (Fig. 3B). As well as the previously described differences in IGFBP secretion pattern we also found in fibroblast conditioned medium an IGFBP at a higher Mr. This we suspect to be ovine BP-3, although confirmation of this will be by blotting with specific anti-serum. Nevertheless, we believe that the data presented in Fig. 3 confirm, especially in the context of IGFBP secretion, the phenotypical and biochemical differences between our fibroblast and chondrocyte cultures.

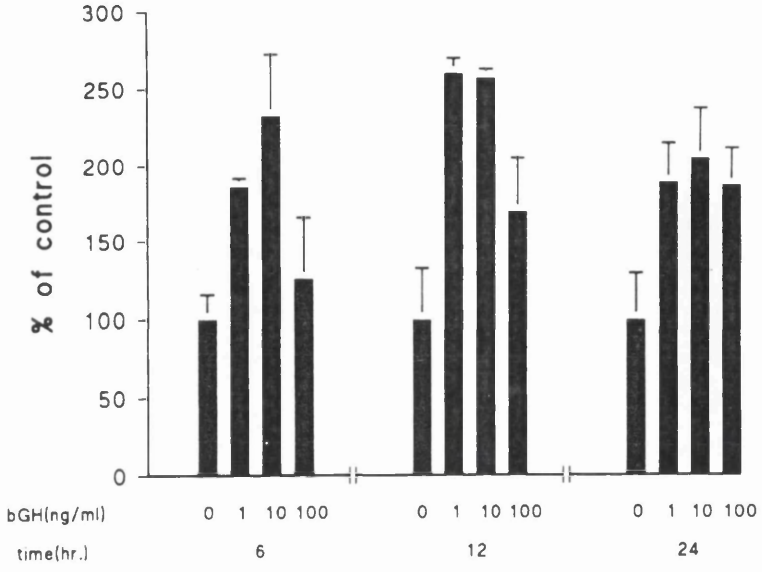

Fig. 2. Solution phase assay for IGFBP concentration in conditioned medium from chondrocyte monolayers. Cells were treated with 0, 1, 10 or 100 ng/ml of bGH and medium was removed after 6, 12 or 24 h for determination of IGFBP levels by solution phase assay. Results are presented as a % of control values (zero bGH) and represent the means ± S.D. of determinations on 3 different wells for each bGH concentration at each time point. For experimental details see Materials and Methods section.

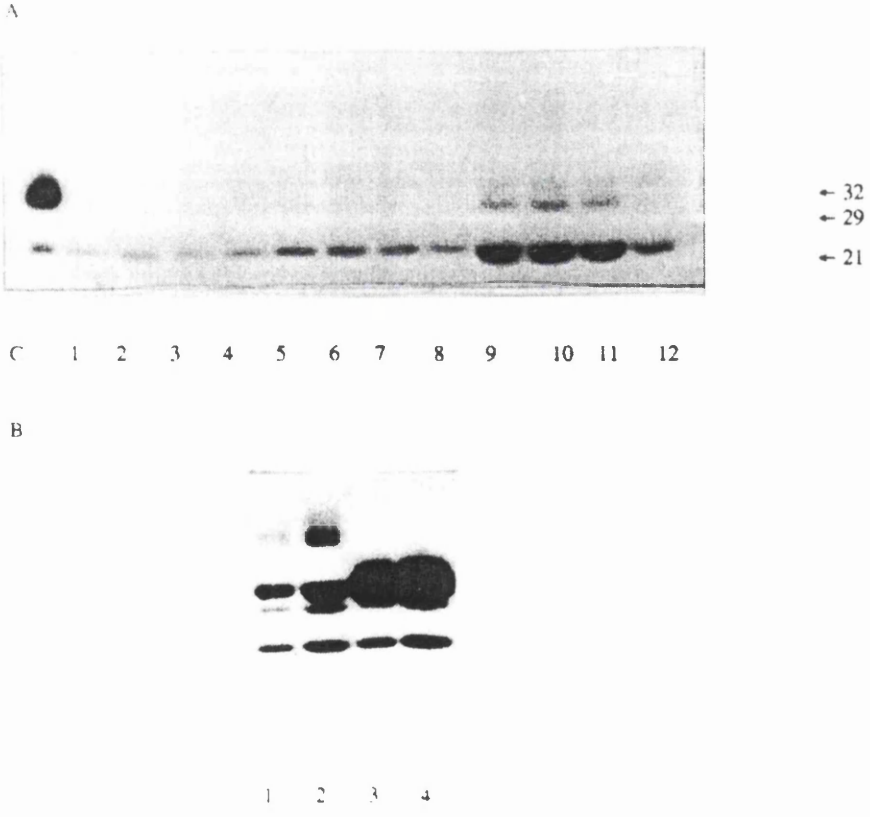

Fig. 3. A Ligand blot analysis of CM from fibroblasts monolayers treated with varying amounts of bGH for either 6 h (lanes 1–4), 12 h (lanes 5–8) or 24 h (lanes 9–12). Concentrations of bGH are 0 (lanes 1, 5 and 9); 1 ng/ml (lanes 2, 6 and 10); 10 ng/ml (lanes 3, 7 and 11) and 100 ng/ml (lanes 4, 8 and 12). As in Fig. 1, lane C contains Clone 9 cell conditioned medium. B Overexposure of a ligand blot analysis of CM from unstimulated fibroblasts (lane 1 = 50 ul; lane 2 = 100 ul) or chondrocytes (lane 3 = 50 ul; lane 4 = 100 ul). Experimental details as described in Materials and Methods section.

In terms of bGH stimulation of IGFBP-2 levels, we show clear effects at each of the 6, 12 and 24 h time points. It is important to note, however, that by 48 and 72 h the effects of bGH were less marked and this should therefore be regarded as an acute effect of bGH in this culture system. In addition, although we describe the results of experiments on chondrocyte monolayers which were grown in culture for 7 days, essentially the same results were obtained in cells grown for 14 days. Assay for IGF-I and IGF-II in acid gel filtration fractions of medium from treated and untreated cells revealed that levels of these peptides were below the sensitivity of the radio-immunoassays used to detect them.

Discussion

There is very little data on the secretion of IGFBPs by chondrocytes in culture. Froger-Gaillard *et al.* [14] working with rabbit articular chondrocytes reported a main IGFBP species of 30 kDa with lesser amounts of a 24 and 20 kDa species. Although GH had no effects on these cells (isolated from 2–3 month old rabbits), these authors also reported the absence of IGFBP-3 in CM cultured by these cells. In a more recent study of bovine chondrocytes derived from adult and foetal articular cartilage and foetal growth plate cartilage and using either high-density monolayer culture or suspension culture in alginate beads, Olney *et al.* [15] reported secretion of IGFBPs of Mr 33 kDa (BP-2) and at 29 and 24 kDa. This is similar to the 32 kD ovine BP-2 and 29 and 21 kDa IGFBPs we report in the present study. It should be noted, however, that this latter study also indicated the presence of BP-3 in CM from these cell types.

Our observation of the up-regulation of IGFBP-2 by bGH is novel and runs somewhat against the current orthodoxy. *In vivo*, hypophysectomy results in a 10–20 fold increase in IGFBP-2 mRNA in liver suggesting a negative regulation by GH *in vivo* [16]. However, the fact that treatment of these hypophysectomised rats with a combination of GH, cortisone, testosterone and T_4 partially reduced hepatic IGFBP-2 mRNA levels towards normal but treatment with GH alone led to a further increase in hepatic mRNA levels over and above that seen in hypophysectomised rats led the authors to conclude that factors other than GH were important in regulating IGFBP-2 mRNA levels. Although the same work identified insulin and nutritional state as important regulators of IGFBP-2, relatively little else is known of *in vivo* and more especially *in vitro* regulation of IGFBP-2. Hopefully, as the promoter region of the IGFBP-2 gene is now being analysed and transcription factor binding sites identified [17, 18], more information will be forthcoming in this area. Although the physiological significance of the local production of IGFBPs in the growth plate remains unknown, two recent studies with a 25 kDa IGFBP purified from amniotic fluid (most likely BP-1) and IGFBP-4 (purified from a human osteosarcoma cell line) were shown to inhibit the basal- and IGF-stimulated growth of chick pelvic cartilage explants [19, 20]. These findings may suggest a somewhat unusual hypothesis that GH (normally considered an anabolic hormone for the growth plate) has an inhibitory effect by stimulating IGFBP-2 release and inhibiting the autocrine/paracrine activity of IGF. It must be borne in mind, however, that physiological effects of IGFBPs are probably specific to the particular form of BP present. Clearly the hypothesis that BP-2 has an inhibitory effect on the growth parameters of these cells requires to be tested directly. Although this area requires further examination such an inhibitory effect of GH would clearly negate any stimulation of IGF-I secretion at the level of the growth plate. One clue to these possible contradictory effects of GH may come from the reported subtle differences in GH action in different regions of the growth plate. This was alluded to briefly in the introduction with GH acting on those cells within the stem cell layer of the growth plate to stimulate differentiation and IGF-I acting on differentiated cells to cause mitogenesis. GH is reported as having little effect on these differentiated cells and even as having an inhibitory effect on colony formation from these cells of the intermediate zone of the growth plate [7]. One possibility is that this inhibitory effect of GH may be mediated via the stimulation of IGFBP-2 production by these cells. Note, however, that in our study we used cells derived from the entire growth plate and therefore the relative abundance of each cell type is unknown. Clearly further experiments with enriched cell populations may prove fruitful. One way to examine these possibilities *in vivo* would be to detect immunohistochemically IGFBP-2 production throughout the region of the growth plate following treatment with GH. As alluded to previously, there is evidence that under some circumstances IGFBPs may potentiate the actions of IGFs. It is possible that GH might therefore enhance the effects of local IGFs by stimulating the production of IGFBPs. In addition given that IGFBP-2 has a higher affinity for IGF-II than IGF-I [21] local changes in IGF-I/IGF-II ratios resulting from increases in IGFBP-2 may also have physiological relevance.

Our solution phase data in Fig. 2 demonstrate a bell-shaped dose response curve for GH stimulation of IGFBP-release, with an optimum effect achieved at around 10 ng/ml and a reduction in IGFBP release at 100 ng/nl rbGH. Such data is consistent with other tissue culture systems which demonstrate this type of response when challenged with varying doses of rbGH [22, 23]. This has been rationalised on the basis of the stoichiometry of interaction between GH and its receptor being 1:2. At high concentrations of GH it is postulated that nonsignalling 1:1 complexes between hormone and receptor may form, leading to a decrease in biological signal. Clearly the exact nature of the GH dose-response curve

obtained will be dependent on the density of GHR present.

In summary, we have demonstrated that rbGH stimulates the secretion of IGFBP-2 by monolayer cultures of ovine chondrocytes. Given the ease with which IGFBPs can be quantitated in conditioned medium and the fact that the growth plate is a classical target tissue for GH action, we believe that such a system may provide a relevant, robust and high throughput *in vitro* bioassay for GH agonists/antagonists. This possibility is currently being examined.

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Amino acids within the extracellular matrix (ECM) binding region (201-218) of rat insulin-like growth factor binding protein (IGFBP)-5 are important determinants in binding IGF-I

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ABSTRACT

The highly conserved N- and C-terminal domains of IGFBPs are believed to participate in IGF binding, but only recently have some of the critical residues in the IGFBP sequence involved in ligand binding been identified. Here we describe two highly conserved amino acids in the C-terminal domain of rat IGFBP-5 that are involved in binding IGF-I. Site-directed mutagenesis was used to produce two mutants, G203K and Q209A, of rIGFBP-5. Relative to wild-type rIGFBP-5, an 8-fold reduction in affinity for human IGF-I was found for recombinant G203K protein in both IGF-I ligand blots and solution phase ligand binding assays, and a 7- and 6-fold reduction for Q209A respectively. This shows that Gly203 and Gln209 in IGFBP-5 are important determinants in binding IGF-I, and due to their complete conservation in all IGFBP sequences, we suggest that they are likely to be involved in binding IGF-I in all six binding proteins. In addition, these two non-basic residues lie within the ECM binding region (201-218) of IGFBP-5, demonstrating that the C-terminus contains partially overlapping IGF-I and ECM binding sites. We therefore propose that heparin binding to basic amino acids in IGFBP-5 between 201-218 may physically occlude subsequent interaction between IGF-I and Gly203/Gln209, and that this may explain previous work of others showing reduced affinity of ECM–bound IGFBP-5 for IGF-I.

INTRODUCTION

The mitogenic and metabolic effects of insulin-like growth factors (IGFs) are mediated mainly through the type 1 IGF receptor, which is found on the surface of most cell types (recently reviewed in Butler *et al.*1997). In turn, the bioavailability of IGFs is regulated by a family of IGF-specific binding proteins (IGFBPs; reviewed in Clemmons 1997). As the affinity constants of the IGFBPs are between 2- and 50-fold greater for binding IGFs than that of the IGF type 1 receptor, they can modulate IGF action by controlling the distribution of IGFs in biological fluids.

Comparisons of IGFBPs 1-6 indicate a high degree of conservation in the N- and C-terminal regions. Of particular note are the largely conserved cysteine residues throughout IGFBPs 1-6 of all species sequenced so far. A non-conserved region separates the N- and C-terminal domains. Both the N- and C-terminal cysteine-rich domains of IGFBPs are believed to participate in IGF binding. This is suggested by the observations that N-terminal cysteine-rich domains of IGFBP-1 (Huhtala *et al.*1986), IGFBP-3 (Sommer *et al.* 1991), IGFBP-4 (Chernausek *et al.* 1995), and IGFBP-5 (Andress *et al.* 1993) and C-terminal cysteine-rich domains of IGFBP-2 (Wang *et al.* 1988; Ho & Baxter 1997) and IGFBP-3 (Binoux *et al.* 1991) all possess residual IGF binding activity. Despite this, to date only a limited number of mutational analyses of IGFBPs have been aimed at the identification of IGF binding domains.

Earlier work has shown that IGFBP-5 binding to extracellular matrix (ECM) results in a reduction in its affinity for IGF-I and enhancement of IGF-I biological actions (Andress & Birnbaum 1992; Jones *et al.* 1993). This was confirmed by experiments

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demonstrating that incubation of IGFBP-5 with glycosaminoglycans (GAGs) resulted in a 17-fold decrease in the affinity of IGFBP-5 for IGF-I (Arai *et al.* 1994). Amino acid substitution and peptide competition experiments have identified a sequence rich in basic amino acids in IGFBP-5 (between Arg201 and Arg218) that is involved in both heparin and ECM binding (Arai *et al.*1996; Parker *et al.* 1996.), and it was proposed that heparin binding to this region may produce a conformational change in IGFBP-5 that results in a reduction of affinity for IGF-I (Arai *et al.*1996). More recently, a systematic mutational analysis of this region has identified Arg207 and Arg214 as the most critical basic amino acids for binding ECM (Parker *et al.* 1998), and, in addition, has shown that mutation of 9 out of the 10 basic amino acids in this region had no effect on IGF-I binding.

Recently, work from several laboratories has begun to identify the specific amino acids in the IGFBP molecule that do contribute to IGF binding. At the N-terminus of bovine IGFBP-2, it was shown that Tyr60 was protected against iodination upon binding of IGFBP-2 to IGF-II (Hobba *et al.* 1996). Subsequently, the same group demonstrated that substitution of tyrosine-60 with alanine or phenyalanine leads to a 4.0- and 8.4-fold reduced affinity for IGF-I respectively, and a 3.5- and 4.0-fold reduced affinity for IGF-II respectively (Hobba *et al.*1998). One suggestion is that the hydroxyl group of Tyr60 may participate in hydrogen bond formation that is important for the initial complex formation with IGF-I, and that this may be a common mechanism for all the IGFBPs, (with the exception of IGFBP-1 which has an alanine residue at the equivalent position). In support of this, others have carried out nuclear magnetic resonance spectroscopy on a bacterially expressed N-terminal fragment of IGFBP-5 (residues 40-92). From this work it was proposed that the primary IGF binding domain comprises Val49, Tyr50 (equivalent to Tyr60 in IGFBP-2), Pro62 and Lys68 to Leu75, where the conserved Leu and Val residues localize in a hydrophobic patch on the surface of the IGFBP-5 protein (Kalus *et al.*1998). However, these researchers also demonstrate that the N-terminal fragments of IGFBP-5 have a 10 to 200-fold lower affinity for IGFs than the full length protein, which agrees well with the reported reduced affinities of other C-terminally truncated fragments of IGFBP-3 (Clemmons 1993) and IGFBP-5 (Andress *et al.*1993). This indicates that other residues at the C-terminus of the IGFBP proteins may be involved in the additional stabilization of IGF complex formation that leads to the very high affinity of binding.

Deletion between residues Cys205 to Val214 at the C-terminus of human IGFBP-4 has recently been shown to cause a 6-fold reduction in affinity for binding IGFs (Qin *et al.*1998). It is conceivable however that deletion of one of the very highly conserved cysteines in this region leads to a major change in the secondary structure of the C-terminal domain, as the six cysteines at the C-terminus of bovine IGFBP-2 (bIGFBP-2) have been shown by Forbes *et al* to form disulphide bonds between consecutive residues (Forbes *et al.* 1998). These latter researchers also demonstrated that truncation of 48 residues from the C-terminus of bIGFBP-2 had no effect on IGF binding (equivalent to residue 200 in human IGFBP-4), suggesting that residues which contribute to IGF binding differ between IGFBP-2 and –4. However, greater reduction in affinity for both IGFs was observed when 63 residues were deleted from the C-terminus of IGFBP-2, with a greater effect on IGF-II binding (up to 80-fold). This therefore identifies a critical region between amino acids 222-236 of bIGFBP-2 involved in binding IGFs (Forbes *et al.* 1998).

This critical region of bIGFBP-2 corresponds to amino acids 201 –216 in the rat IGFBP-5 (rIGFBP-5) sequence, which lies within the basic heparin binding region of the protein. Alignment of amino acid sequences for all six binding proteins from several species revealed that there are completely conserved glycine and glutamine residues (Gly203 and Gln209 in rIGFBP-5) within this region (Fig 1A), which, in turn, suggests an important conserved function for these amino acids. Using site directed mutagenesis, we have made two mutants of rIGFBP-5 (G203K and Q209A), and examined their affinity for binding human IGF-I (hIGF-I). We report here that the individual mutations both cause an approximately 8- to 7-fold reduction in affinity for hIGF-I as shown both by IGF-I ligand blotting and solution phase ligand binding assays. The implications of these findings for IGFBP-5 interaction with both IGF-I and ECM are discussed.

MATERIALS AND METHODS

Site-directed mutagenesis

The full-length cDNA for rIGFBP-5 in pGEM®-7*zf* (Promega), containing both initiator and signal peptide, was kindly provided by Dr S. Guenette, Los

Table 1. Affinity of wtIGFBP-5, G203K and Q209A for hIGF-I

	Western Analysis		Scatchard Analysis	
Protein	Estimation of protein concentration (μg/ml)	Affinity for hIGF-1: % wt	Estimation of protein concentration (μg/ml)	Affinity for hIGF-I: K_D (nM)
wt BP-5	5.5	100	6.3	0.154
G203K	6.9	12.5	8.1	1.25
Q209A	7.8	15.0	11	0.84

K_D values were obtained by Scatchard analysis of binding data as described in the Methods section.

Angeles. It was subcloned into the EcoRI restriction site of pFastBac® (Gibco-BRL) for eventual expression in the baculovirus system. Site-directed mutagenesis was then performed at two separate sites on the rIGFBP-5 cDNA using the Gene Editor® *in vitro* mutagenesis system (Promega). For the G203K mutation, the oligonucleotide 5'-CCAACTGTGACCGCAAAAAATTCTACAAGA GAAAGC-3' was used, which created a unique ApoI restriction site, and for Q209A it was 5'-GATTCTACAAGAGAAAGGCATGCAAGCCTTC TCGTGG-3', which created a unique SphI restriction site. The products of the initial mutagenesis reactions were transformed into *E. coli* ES1301 *mutS*, a repair minus strain, to avoid selection against the desired mutations. Following mutagenesis, the DNA was

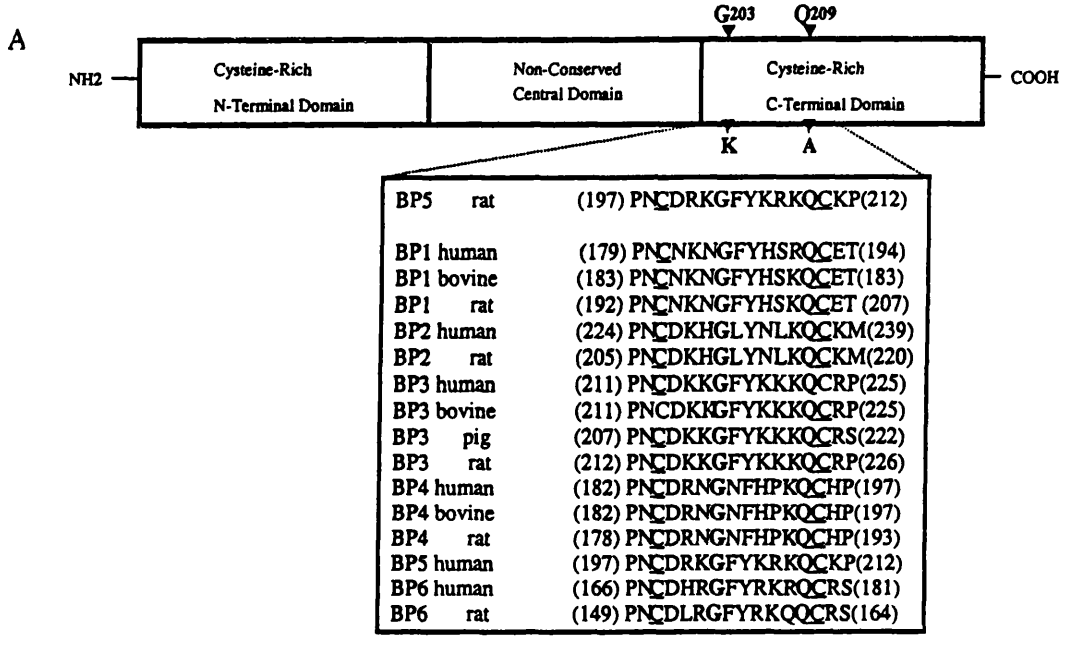

FIGURE 1

A. C-terminal amino acid sequences of IGFBP. At the top is a schematic representation of the three structural domains of the IGFBPs. The relative location of the conserved glycine and glutamine residues are shown above the C-terminal domain, and the substituted amino acids below. The box underneath shows a line-up of the amino acid sequences for this C-terminal region in all six IGFBPs from several different species. The residue number at the start and end of each sequence is indicated in brackets. The conserved cysteines are underlined and the G203 and Q209 are in bold. B. Amino acid sequence of the 201-218 region of IGFBP-5. Basic amino acids are underlined and Gly203 and Gln209 are in bold.

maxi-prepped using the Qiagen Plasmid Maxi Prep Kit and manually sequenced using the T7 Sequenase™ version 2.0 DNA sequencing kit (Amersham Life Sciences), which verified that the correct mutations were present.

Baculovirus expression

Expression of recombinant IGFBPs in the insect cell-baculovirus system was carried out using the Bac-to-Bac® system (Gibco-BRL), which employs the techniques of Lucklow *et al.* 1993 to produce recombinant virus. Conditions for expression were the same as that in the manufacturer's guide, with a chosen multiplicity of infection of 5. Sf9 insect cells (*Spodoptera frugiperda*) were grown in Sf-900 II medium (Life Technologies). 50 ml suspension cultures were grown in spinner flasks (Techne) and culture supernatant containing secreted IGFBP protein was harvested 24 to 28 hours after infection, depending on the optimum level of expression of the individual protein. Supernatants were concentrated using 20 ml Ultrafree-20® (Millipore) columns and aliquots were frozen in liquid nitrogen and stored at –70 °C.

IGF-I Ligand Blotting

Proteins were electrophoresed on 12% acrylamide SDS gels under non-reducing conditions and subsequently transferred to nitrocellulose membranes. Ligand blots were performed according to the method described by Hossenlopp *et al.* 1986 , using IGF-I (GroPep) radiolabelled to a specific activity of approximately 100 μCi/μg. Following ligand analysis the same blots were used for immunodetection.

Immunoblotting

Western blotting of wt IGFBP-5, G203K and Q209A was performed to monitor protein loading of samples which had previously been subjected to ligand blot analysis. Immunodetection was with a sheep antibody raised in our laboratory to baculovirus-expressed wt rIGFBP-5 protein provided by Dr Sean Guenette (John Wayne Cancer Institute, Los Angeles). First antibody was used at a dilution of 1:2000 and anti sheep-HRP conjugate at 1:20,000. Reactivity was determined using Enhanced Chemiluminesence (ECL) (Amersham) and densitometric analysis (Molecular Dynamics).

Quantitation of wt IGFBP-5, G203K and Q209A in baculovirus supernatants was achieved by Western blot/densitometry using as standard wt IGFBP-5 supplied by Dennis Andress (Veterans Affairs Medical Center, Seattle). Care was taken to operate with standards and samples within the linear range of the ECL/Film response methodology. Using this technique protein concentrations of 5.5, 6.9 and 7.8 μg/ml were found for wt IGFBP-5, G203K and Q209A respectively (Table 1).

Solution Phase Assay of IGFBP activity

Activity of wt IGFBP-5, G203K and Q209A were assessed in solution by the method described by Conover *et al.* 1989. Amounts of wt, G203K and Q209A proteins were present in assay tubes such that approximately 20% of added ^{125}I-IGF-I (25-30,000 cpm) was bound. Affinity of wt and mutated IGFBP-5 proteins was assessed by Scatchard analysis of binding curves obtained following addition of increasing concentrations of unlabelled IGF-I. Protein concentrations as estimated by Scatchard are 6.3, 8.1 and 11 μg/ml for wt IGFBP-5, G203K and Q209A respectively (Table 1).

RESULTS AND DISCUSSION

Two amino acids in the C-terminus of IGFBPs (Gly203 and Gln 209 in IGFBP-5) are completely conserved between all six binding proteins from all species sequenced to date (Fig 1A). In this work, we demonstrate that these residues in rIGFBP-5 are important determinants in binding IGF-I. Wild-type IGFBP-5 and the two mutants G203K and Q209A were probed with ^{125}I- labelled IGF-I in ligand blots, and the same blots were subsequently probed with a sheep anti-IGFBP-5 polyclonal antisera (Fig 2A). The IGFBP-5 doublet produced by expression in the baculovirus system is a common feature from several laboratories (personal communications Dr D. Andress, Dr S. Guenette), including commercially available hIGFBP-5 (Austral Biologicals), and this may be the result of inefficient cleavage of the mammalian signal peptide during secretion. Nevertheless, BiaCore analysis of the wt IGFBP-5/hIGF-I and –II interaction recorded a single association and dissociation event (data not shown), suggesting that the IGFBP-5 doublet represents a single species with respect to IGF-I binding. Also, the strong recognition of the mutant proteins with our IGFBP-5 antibody demonstrates their immunological integrity. Densitometry was carried out on ligand and

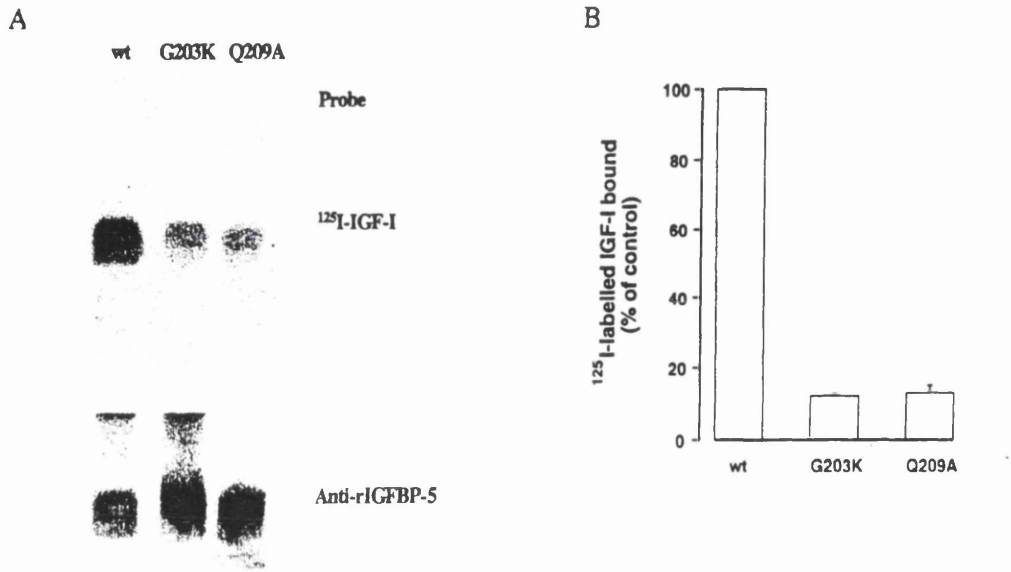

FIGURE 2. Western analysis of wild-type and mutant protein.
A. Both autoradiographs were produced from a single western blot. The upper autoradiograph is a ligand blot of wt rIGFBP-5 protein and the two mutant proteins G203K and Q209A probed with ^{125}I-hIGF-I. The lower autoradiograph is the same blot subsequently probed with sheep anti-rIGFBP-5 polyclonal antisera.
B. Histogram showing the results of a densitometric analysis of the upper autoradiograph in 2A (n = 2 ± range).

western blots from two separate experiments and the ratio between the strength of signal in ligand and western blot determined (Fig 2B; Table 1). This demonstrated that, relative to wt IGFBP-5, G203K and Q209A have an 8- and 7-fold reduction in affinity for IGF-I respectively.

This is in very strong agreement with the K_d values derived from solution phase binding assays for these three proteins (Fig 3; Table 1). The K_d of wt IGFBP-5 in our experiments is also in agreement with previous results using mammalian and yeast expressed protein (Kiefer *et al.*1992), but we demonstrate that, relative to wt IGFBP-5, G203K and Q209A have a 8- and 6-fold reduction in affinity for IGF-I respectively (Table 1). Forbes *et al* 1998 demonstrated up to an 80-fold reduction in IGF-II affinity when the equivalent amino acids (222-236) were deleted from bIGFBP-2. This larger reduction in affinity may be a result of differential IGF-I and –II recognition. Alternatively, loss of both conserved amino acids may explain the larger effect. The affinity of a double mutation in rIGFBP-5 (G203K, Q209A) for both IGF-I and -II is the subject of ongoing research in our laboratory. Note, however, that the C-terminal truncated bIGFBP-2 proteins may have lost other residues involved in IGF binding. Indeed, smaller truncations did appear to alter IGF binding specificity selectively, suggesting that other more C-terminal residues may be involved in differential IGF-I and –II recognition (Forbes *et al.* 1998).

A myriad of functions has now been ascribed to the basic amino acid rich region between Arg201 and Arg218 in IGFBP-5. In addition to heparin and ECM binding (Arai *et al.* 1996; Parker *et al.*1996), this region has been implicated in binding to the acid labile subunit (Twigg *et al.* 1998), the putative IGFBP-5 receptor (Andress 1998), plasminogen activator inhibitor-I (Nam *et al.* 1997), and has even been postulated to have strong homology to a nuclear localization signal (Schedlich *et al.* 1998) and the DNA-binding domains of several transcription factors (Schedlich *et al* 1998; Turner & Tijan 1989). Recently, a systematic mutational analysis of this region, using either single or combined substitutions of basic amino acids, has identified the critical residues involved in ECM binding (Parker *et al.* 1998). While some of these mutations displayed major reductions in ECM binding, they were shown to have little effect on IGF-I affinity, and it was concluded that the 201-218 region was not involved in binding IGF-I.

It is, therefore, of major significance that two of the non-basic amino acids lying within this region have now been shown to be involved in IGF-I binding. In the linear sequence, Gly203 and Gln209 flank a

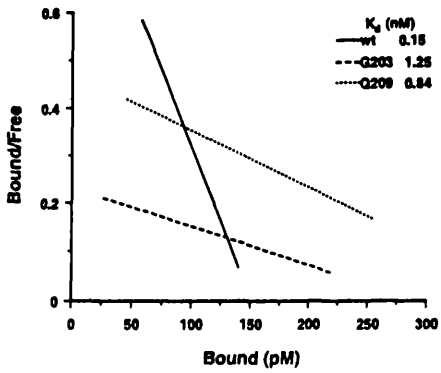

FIGURE 3. Regression lines drawn through the data obtained by Scatchard analysis of hIGF-I binding to wt IGFBP-5 (solid line: y = -0.0063x + 0.95 (r = -0.94)), G203K (dashed line: y = -0.0008x + 0.23 (r = -0.96)) or Q209A (dotted line: y = -0.0012x + 0.47 (r = -0.77)). IGFBPs were present at 1.1, 6.9 and 7.4 ng/tube for wt, G203K and Q209A respectively. K_d values were determined from these plots and are as indicated in the Figure. This experiment was performed twice and similar results were obtained in each instance.

critical residue involved in ECM binding (Arg207), and, in turn, are themselves flanked by other basic residues which play a part in this interaction (Fig 1B). In addition, a helical wheel prediction of the 201-218 region of IGFBP-5 (Parker *et al.*1998), places Gly203 adjacent to the critical ECM binding residues, while Gln209 is flanked by two further basic residues elsewhere in the wheel. Thus, we have demonstrated that IGF-I and ECM binding sites in IGFBP-5 are in very close proximity and may partially overlap.

This has important implications for the function of IGFBP-5. Heparin or ECM binding to the basic amino acids in the 201-218 region of IGFBP-5 may physically occlude any subsequent interaction between Gly203/Gln209 and IGF-I. This would offer an alternative explanation for the observed reduced affinity of ECM/GAG bound IGFBP-5 for IGF-I (Andress *et al.* 1992; Jones *et al* 1993.; Arai *et al.*1994), rather than a heparin induced conformational change in IGFBP-5 which alters an IGF binding domain elsewhere in the protein. The lower affinity of ECM-bound IGFBP-5 for IGF-I may be biologically important for obtaining the correct balance between soluble IGF-I available for binding the type 1 IGF receptor and a reservoir of immobilised IGF-I protected from proteolysis while bound to the ECM.

In summary, we have identified two highly conserved C-terminal residues in IGFBP-5 involved in IGF-I binding. Due to the complete conservation of these two amino acids in all IGFBP sequences, we suggest that they are likely to be involved in binding IGF-I in all six of the binding proteins. Overlap of these residues in IGFBP-5 with other amino acids between 201-218, that have been shown to be involved in ECM binding, suggests their involvement in the interaction between IGFBP-5, ECM and IGF-I.

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Characterization of the IGF axis in a rat liver-derived epithelial cell line

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Summary We have used the techniques of chemical cross-linking, Western blotting and immunoprecipitation-ligand blotting to demonstrate that insulin-like growth factor binding protein-2 (IGFBP-2) is associated with plasma membranes of an epithelial cell line derived from rat liver as well as being secreted into the medium by these cells. We demonstrate that these cells secrete IGF-I, but not IGF-II into serum free medium. Evidence from signalling, cell proliferation and cross-linking experiments indicate that these cells also express cell surface IGF-I receptors. Dose–response experiments indicate an enhanced biological activity of the IGF-I analogue des (1–3) IGF-I compared to wild-type IGF-I in both acute signalling experiments and longer-term (24 h) mitogenic assays. As this IGF-I analogue has lower affinity for IGFBPs, we believe that in this cell culture system, activity of IGF-I may be attenuated in the long and short term by the accumulation of IGFBP-2 in conditioned medium and by the presence of IGFBP-2 associated with the cell membrane and/or ECM.

Key words: rat liver epithelial cell line, insulin-like growth factors, insulin-like growth factor binding protein.

INTRODUCTION

The insulin-like growth factors (IGF-I and -II) are small polypeptides which have important metabolic and growth promoting actions (reviewed in ref. 1). In biological fluids only a small proportion of the IGFs exist in a free form, with the major fraction of circulating IGFs being bound to high-affinity carrier proteins – the IGF binding proteins (IGFBPs). To date, six such binding proteins have been identified and cloned for both rat and humans[2]. In addition, a family of four low-affinity IGF binding proteins has recently been described[3]. The six high-affinity IGFBPs(1–6) are characterized by a high degree of structural similarity. This is typically apparent by the conservation of cysteine residues in the primary structure, all of which are involved in disulphide bond formation and which are in turn believed to be important in forming a molecular 'scaffold' for these proteins. Although no X-ray crystallographic structure is available for any of the IGF binding proteins, recent evidence from peptide mapping and proteolysis studies[4] suggests a three-domain model for these proteins with cysteine-rich N- and C- terminal domains being connected by a central hinge region.

In a functional sense, IGFBPs have traditionally been viewed as a reservoir of polypeptide growth factor, buffering tissues from the hypoglycemic effects of large circulating levels of IGF-I. Recent molecular and cellular studies, however, have led to a refinement of this view and have postulated activities of IGFBPs, which have included hormone-independent effects[5]. One major area of research has been developed following the reports of interaction of IGFBPs with various elements of the extracellular matrix and cell membranes[6,7]. Interactions with ECM are believed to take place via positively-charged consensus heparin-binding sequences present in all binding proteins except IGFBP-4. Such binding is believed to affect the biological properties of the IGFBP proteins by altering affinities for IGFs and by protecting BPs from degradation by locally produced proteases[8,9]. Clearly this method of modulating IGFBP activity could have important biological implications in regulating the supply of IGF polypeptide to target tissues.

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For IGFBP-1, interaction with cell membrane integrin $\alpha_5\beta_1$ (via a C-terminal RGD sequence in the binding protein) has been reported, and IGF-independent effects of BP-1 on cell migration were documented[6]. In addition, IGFBP-3 and IGFBP-5 have also been reported to associate with cell membrane protein or glycosaminoglycan receptors[10,11]. Like IGFBP-1, IGFBP-2 also possesses a C-terminal RGD integrin binding motif, although to date no interaction with this class of cell surface binding molecule has been proposed for this particular binding protein. Notwithstanding this, limited reports have suggested that IGFBP-2 is indeed identifiable in plasma membrane preparations[12–14]. In order to provide further support for this phenomenon and address its biological significance, we have investigated the association of IGFBP-2, secreted by a rat liver epithelial cell line, with membranes derived from monolayer culture of these cells. By the use of various techniques we clearly demonstrate such an association and provide further support for the hypothesis that IGFBP-2 binds to plasma membranes. Further, we demonstrate the secretion of IGF-I by these cells and the presence of functional IGF-I receptors in cell membranes. In addition, we describe experiments which suggest an important role for secreted, and perhaps membrane-associated IGFBP-2 in the autocrine regulation of the cell response to IGF-I.

MATERIALS AND METHODS

Materials

The rat liver epithelial cell line (Clone 9) was obtained from the European Collection of Animal Cell Cultures (ECACC Cat No CRL1439) at Salisbury, UK[15]; reagents for tissue culture were from Gibco, (Inchinnan, UK); tissue culture plastic was from Costar (Bucks, UK); mini-gel electrophoresis and blotting equipment was from BioRad, Herts, UK; BCA protein reagent was from Pierce (Chester, UK); Enhanced Chemiluminescent Reagent (ECL), Hyperfilm, Hybond-C and PVDF membranes were from Amersham (Bucks, UK); MTT (thiazolyl blue) was from Sigma; rabbit polyclonal anti-bovine IGFBP-2 and anti-phosphotyrosine monoclonal antibody 4G10 were from TCS Biologicals (Bucks, UK); rabbit polyclonal anti-human IGF-I was a gift from NIADDK (Bethesda, USA) and rabbit polyclonal anti-human IGF-II was from GroPep Pty (Adelaide, Australia); horse radish peroxidase (HRP) conjugated secondary antibodies were from Sigma; pansorbin was from Calbiochem (Nottingham, UK, Cat No 507861); h IGF-I, hIGF-II and des (1–3) IGF-I were from GroPep Pty (Adelaide, Australia); bovine insulin was from Sigma. All other reagents were of analytical grade. Baculovirus derived recombinant rat IGFBP-2 was produced in our laboratories.

METHODS

Cell culture and plasma membrane preparation

Clone 9 cells were grown in Hams F12 containing 2 mM glutamine, 10% FCS, Penstrep and plated in 10 cm Petri dishes. When confluence was reached, cells were washed with Hanks Balanced Salt Solution (HBSS) and serum-starved overnight in serum free medium (SFM – Hams F12 / 0.1% BSA/ 2 mM glutamine). Enriched cell membrane preparations were prepared as described by Woodward *et al.*[16]. Monolayers were washed twice with ice cold PBS and cells were detached from plates in hypotonic buffer [1 mM EDTA, 1 mM Na bicarbonate (pH 7.4) 2 mM PMSF, 3 μM leupeptin, 16 μM aprotinin, 500 μM $NaVo_4$] The cell suspension was homogenized (Polytron 10 s) and centrifuged at 1000 *g* at 4°C for 10 min. The supernatant from this first spin was centrifuged at 15 000 *g* for 5 min. To produce an enriched plasma membrane preparation, the 15 000 *g* supernatant was centrifuged at 50 000 *g* for 90 min. Membranes were resuspended in 10 mM Tris pH 7.4 in the presence of protease inhibitors, the protein concentration was determined with BCA protein assay and aliquots were stored at – 20°C prior to further analysis.

Western and ligand blotting

Concentrated samples of the enriched plasma membrane preparations were subject to electrophoresis on 12% non-reducing SDS gels and transferred to Hybond C membranes. Ligand blots were performed essentially according to the method described by Hossenlopp *et al.*[17] Briefly, membranes were washed in 3% NP_{40} in Tris buffered-saline [TBS – 10 mM Tris 60 mM NaCl (pH 7.4)] for 30 min, TBS – 1% BSA for 2 h and TBS – 0.1% Tween (TBS-T) 20 for 10 min. Membranes were hybridized overnight with ^{125}I-IGF-I (~1.5 × 10^6 cpm) in 1–2 ml TBS – 1% BSA, 0.1% Tween 20 at 4°C. The membranes were then washed in TBS -T (3 × 15 min) and 2 × 15 min in TBS. Membranes were dried and exposed to X-ray film for periods of up to 10 days. For Western blots, membranes were blocked in 3% BSA-TBS-T and incubated with a rabbit polyclonal αIGFBP2 Ab 1:2000 (in 1% BSA-TBS-T) for 1 h, washed in TBS-T (3 × 15 min), incubated with an αrabbit-HRP (1:5000 in 5% non-fat dried milk in TBS-T) for 1 h and washed (3 × 15 min; TBS-T) again. Protein bands were visualized by ECL detection.

Immunoprecipitation of membrane associated IGFBP-2

The immunoprecipitation of solubilized, Clone 9 plasma membranes was performed as a modification of a method previously described[13]. 800 μg of plasma membranes were solubilized by treatment for 1 h at 4°C in 400 μl buffer A (50 mM Tris pH 7.4, 150 mM NaCl, 0.01% NaN_3, 0.8 mg/ml pepstatin, 2 mg/ml aprotinin, 2 mg/ml

leupeptin, 1% Triton x-100, 1% Na cholate). 400 µl of buffer B (20 mM Tris HCl pH 7.5, 10 mM $MgCl_2$, 0.2% BSA) was added and samples centrifuged at 28 500 × g for 30 min at 4°C. The supernatants were transferred to fresh tubes and rotated overnight at 4°C with 10 µl anti-bovine IGFBP-2. The antibodies were precipitated by the addition of 50 µl Pansorbin and centrifugation at 6000 × g for 10 min. The resultant pellet was washed three times with buffer A and resuspended in 50 µl of 2X non-reducing sample buffer, left on ice for 30 min, then boiled for 3 min. Samples were analysed by ligand blotting with ^{125}I-IGF-I as described above.

Detection of IGF-I and IGF-II in Clone 9 cell conditioned medium

Confluent monolayers of Clone 9 cells (4×10^7 cells) were allowed to condition serum and protein free medium (~130 ml) over a period of 24 h. The volume of medium was reduced in two stages by freeze-drying to approximately 5 ml and dialysed extensively against 10 mM Na phosphate (pH 7). Samples (10 µl) of concentrated, dialysed medium were subject to non-reducing SDS-PAGE (16% polyacrylamide gels) along with IGF-I and IGF-II standards before transfer to PVDF membranes. Blots were probed with rabbit anti-human IGF-I (diluted 1:2000 in TBS-T) before development with anti-rabbit HRP conjugate (1:5000) in TBS-T and ECL reagent. IGF-II was detected with a rabbit polyclonal anti-human IGF-II in the same manner on parallel blots.

Effect of hormones on tyrosine phosphorylation in Clone 9 cells

Confluent monolayers of Clone 9 cells in 10-cm tissue culture dishes were starved overnight in serum free media. Prior to hormonal stimulation, media was removed and the cell monolayers were washed once with HBSS. Cells were then treated with either IGF-I, des (1–3) IGF-I or IGF-II (1–100 ng/ml) or insulin (100 or 1000 ng/ml) in 5ml of fresh serum free medium for periods of 2, 5 or 10 min. At the end of this incubation period, medium was removed, monolayers washed twice with ice-cold PBS and cell lysates were prepared as described previously[18]. Lysates were run on SDS-PAGE (7.5% gels) under reducing conditions and transferred to Hybond C or PVDF membranes. The profile of tyrosine-phosphorylated proteins was determined by immunoblotting with the anti – pY Mab 4G10 (1:5000 in TBS-T) with detection by anti-mouse HRP conjugate (1:20 000 in TBS-T) and ECL

Cell proliferation assay

To measure the mitogenic response of Clone 9 cells to different polypeptide hormones, cells were trypsinized from stock cultures and seeded in 96-well tissue culture plates at a density of 6×10^3 cells/well in 50 µl of Hams F12/ 0.1%FCS/PenStrep. After overnight incubation (37°C, 5% CO_2), cells were treated for 24–48 h with a further 50 µl of Hams F12/ 0.1%FCS/ Penstrep containing various concentrations of IGF-I, IGF-II, des(1–3) IGF-I or insulin as described in Fig. 5 (A,B). At the end of the incubation period, 10 µl of stock MTT (3-[4,5-Dimethylthiazol-2-yl]-3,5-diphenylformazan) – 5 mg per ml in RPMI 1640 medium w/o Phenol Red was added to each well, and incubation was continued for a further 3 h. Following solubilization of dye by the addition of 150 µl of 0.05 M HCl in isopropanol, absorbance of microtitre wells was determined at 620 nm. Mitogenic/metabolic activities of added polypeptides was measured by increased absorbance at this wavelength (see Fig. 5 A,B).

Cross-linking

10^6 cpm ^{125}I-IGF-I or ^{125}I-IGF-II were incubated with 400 µg membrane protein in a total volume of 1 ml of reaction buffer (RB) – 5 mM Tris.Cl (pH 7.4) containing 0.5% (w/v) fatty acid free BSA– in the presence or absence of competing unlabelled hormone [IGF-I, IGF-II, des (1–3) IGF-I or insulin: 1 µg/tube]. Reactions were rotated at RT for 3 h followed by centrifugation (10 000 *g*, 10 min). Supernatants were discarded, pellets washed by resuspension in 1 ml of RB and re-centrifuged. Finally, pellets were resuspended in BSA-free RB and cross-linking was achieved by the addition of 50 µl of 40 mM DSS in anhydrous DMSO and incubation at 4°C for 15 min. The reaction was quenched by the addition of 200 µl 50 mM Tris.Cl (pH 7.4) 5 mM EDTA. Centrifugation was repeated as described above, membrane pellets resuspended in SDS sample buffer boiled for 3 min and resolved on 12% gels under reducing conditions. Gels were Coomassie Blue stained, dried and used for direct autoradiography.

Iodination

IGF-I and IGF-II were iodinated by the Iodogen coated tube method to a specific activity of ~100 µCi/ug[19].

RESULTS

In initial experiments to characterize components of the IGF axis in the Clone 9 cell line, we examined the receptor profile of the cells. In Fig. 1 we show the results of cross-linking experiments with preparations of Clone 9 cells plasma membranes and ^{125}I-IGF-I. Two major bands were detected at Mrs of approximately 36 and 120 kDa. The patterns of competition during cross-linking experiments indicated that the higher Mr band represented the α-subunit of the IGF-I receptor and indeed Clone 9 cells respond to IGF-I (see below). The lower Mr band was competed on co-incubation with IGF-I and IGF-II, but not with des (1–3) IGF-I or insulin. Such a pattern of com-

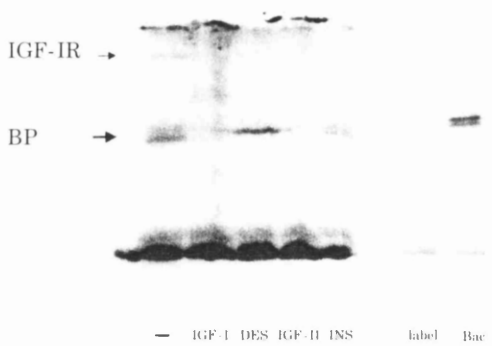

Fig. 1 Cross-linking of ^{125}I-IGF-I to Clone 9 cell plasma membranes. 10^6 cpm ^{125}I-IGF-I was cross-linked to 400 μg of Clone 9 cell plasma membranes, as described in Materials and Methods. Incubations were performed in the absence of competitor or in the presence of 1 μg IGF-I, des(1–3) IGF-I, IGF-II or insulin as indicated. Labelled bands were detected by phosphorimager. The location of putative cross-linked IGFBP (~36 kDa) and the α-subunit of the IGF-I receptor are indicated by broad and thin arrows respectively. Control experiments show the pattern obtained after cross-linking ^{125}I-IGF-I to baculovirus-derived recombinant rat IGFBP-2 (Bac) or cross-linking labelled IGF-I alone (label). This experiment was repeated three times.

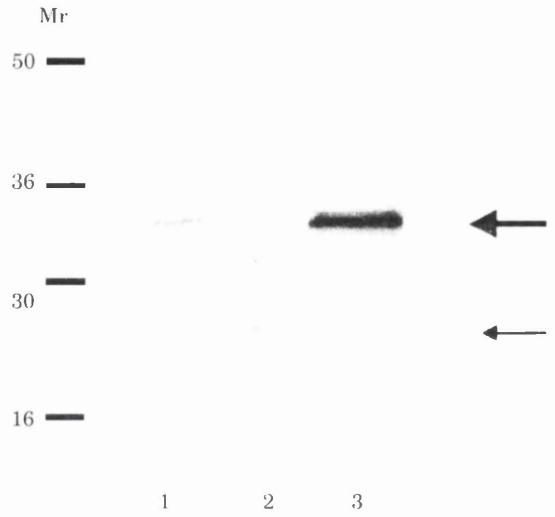

Fig. 2 Proteins in solubilized Clone 9 cell membranes were separated by electrophoresis and transferred to Hybond C membranes. Ligand blotting was performed with ^{125}I-IGF-I and identified an IGFBP (lane 2), which co-migrated with recombinant baculovirus derived rat IGFBP-2 (lane 3). To confirm the identity of this IGFBP, solubilized membrane preparations were subject to immunoprecipitation with polyclonal rabbit anti-bovine IGFBP-2, as described in Materials and Methods. Subsequent blotting of immunoprecipitates with ^{125}I-IGF-I confirmed the presence of IGFBP-2 in cell membranes (lane 1: thick arrow). Note the presence of a smaller Mr IGF-I binding species present in non-immunoprecipitated membranes (lane 2: thin arrow). This experiment was repeated three times with identical results in each case.

petition is characteristic of the IGFBP family. Cross-linking experiments with ^{125}I-IGF-II also indicated the presence of this IGFBP species and demonstrated an identical pattern of competition to that observed with ^{125}I-IGF-I cross-linking. In addition, a very high Mr band is apparent which shows characteristics of the IGF-II/mannose-6-phosphate receptor (data not shown). These data therefore suggest that Clone 9 cells express IGF-I receptors and probably also IGF-II receptors. Although we did not perform any cross-linking experiments with ^{125}I-insulin, the results of our hormone stimulation experiments (see later) indicate that insulin receptors are either missing or present at a very low abundance.

Repeatedly during the course of these experiments with plasma membrane preparations, it was evident that a lower Mr band (~30–40 kDa) was specifically labelled following either ^{125}I-IGF-I or ^{125}I-IGF-II cross-linking. As cross-linked IGF-IGFBP complexes would be predicted to have an Mr of this order and as competition analysis suggested that this species may be a member of the IGFBP family, we conducted further experiments to test the hypothesis that an IGFBP(s) was associated with the plasma membrane of Clone 9 cells. In Fig. 2 we present the results of a ligand blotting experiment, where solubilized membrane preparations have been probed with ^{125}I-IGF-I. Two IGFBP species are evident (lane 2). The higher Mr binding protein migrates at the same location as baculovirus-derived rat IGFBP-2, and along with others we have demonstrated previously that Clone 9 cells produce IGFBP-2, and experiments to date suggest that this is the only binding protein species present in medium conditioned by this cell line.[20–22] We hypothesized, therefore, that this membrane associated IGFBP could also be IGFBP-2. To confirm this, we performed immunoprecipitation experiments with solubilized Clone 9 cell membranes and anti-bovine IGFBP-2 antiserum. This antiserum recognizes IGFBP-2 from several species (including rat) with only minimal cross-reactivity with other IGFBPs[23]. Ligand blot of the resultant immune complex identified the cell-associated IGF binding protein as IGFBP-2 (lane 1). The smaller Mr IGF binding protein fragment was not immunoprecipitated with anti-BP2. This smaller Mr fragment does not appear consistently, and its identity is currently unknown.

We were interested in examining whether IGFBP-2 could potentially affect the response of Clone 9 cells to exogenous IGF-I. Preliminary evidence from our cross-linking studies indicated the presence in cell membranes of IGF-I receptors. We therefore conducted hormone stimulation experiments using cultures of Clone 9 cells. We designed two different experimental models to work within. Initially, we examined the acute effects of IGF-I, des (1–3) IGF-I, IGF-II and insulin in the stimulation of protein tyrosine phosphorylation in Clone 9 cells over a short time course. In Fig. 3 we present results of

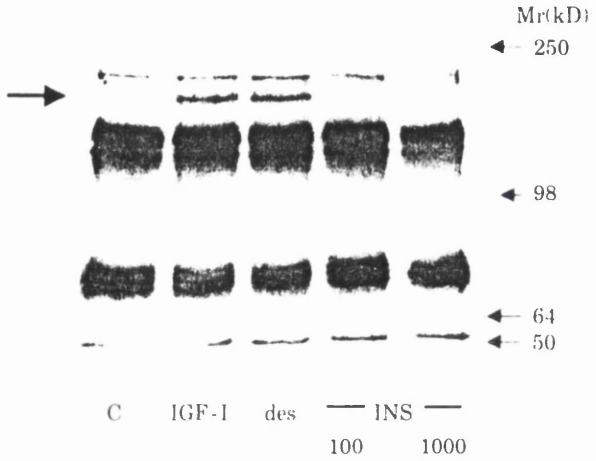

Fig. 3 Clone 9 cell monolayers were treated with 100 ng/ml IGF-I, des (1–3) IGF-I or insulin (100 and 1000 ng/ml). After preparation of cell lysates, tyrosine phosphorylated proteins were detected by blotting with the anti-phosphotyrosine antibody 4G10 as described in Materials and Methods. The arrow indicates the IGF-I and des (1–3) IGF-I specific induction of a ~180 kD tyrosine-phosphorylated protein. Insulin at 100 or 1000 ng/ml had no effect on the level of tyrosine phosphorylation of this protein. This experiment was repeated three times.

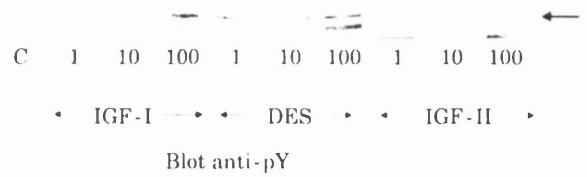

Fig. 4 Dose-dependency of induction of 180 kDa tyrosine phosphorylated protein by IGF-I, des (1–3) IGF-I and IGF-II. Confluent layers of Clone 9 cells were treated with polypeptide hormones (1, 10 or 100 ng/ml) for 5 min. After preparation of cell lysates, patterns of tyrosine phosphorylated proteins were detected as for Fig. 4 by blotting with 4G10. Arrows indicate the dose-dependent stimulation of tyrosine phosphorylation of 180 kDa protein by both IGF-I and the des (1–3) analogue. This experiment was repeated twice.

anti-phosphotyrosine blots of cell lysates following treatment of cultures with IGF-I, des (1–3) IGF-I or insulin. IGF-I and des (1–3) IGF-I (100 ng/ml for both) were found to stimulate the tyrosine phosphorylation of a protein of Mr ~180 kD, whereas insulin, even at two pharmacological doses, had little effect on the tyrosine phosphorylation of this protein. Although we have postulated that this protein is a member of the insulin receptor substrate family (IRS), we have to date, been unable to precipitate this species with commercial anti-IRS 1 antisera. However, this data confirms the presence in Clone 9 cells of functional IGF-I receptors and may suggest a relative paucity of insulin receptors, (although this would require further confirmation by, for example binding and/or cross-linking studies with radio-labelled insulin). In addition, dose–response studies with both IGF-I and des (1–3) IGF-I indicated that the latter hormone (which binds poorly to IGFBPs) was more active in stimulating the tyrosine phosphorylation of this protein (Fig. 4) at concentrations of 10 and 100 ng/ml (see Discussion)

These results were confirmed both in terms of activity profiles and dose-responses in a separate bio- assay system where the metabolic/mitogenic activity of hormones was tested by the MTT assay system (Fig. 5). This formazan dye-based method for the examination of the mitogenic/metabolic activities of hormones has been used previously in the context of IGF ligands[24]. Again, IGF-I and des (1–3) IGF-I demonstrated considerable activity in this assay, whereas insulin (Fig. 5A) and IGF-II (Fig. 5B) had stimulatory effect only at higher concentrations (which probably indicates cross-reactivity of these hormones at the IGF-I receptor). These results paralleled those of the signalling experiments described in Figs 3 and 4 in respect of the activity of IGF analogues. Indeed, we were interested to note the apparent increased activity of the des (1–3) IGF-I analogue in this metabolic assay, which demonstrated a much higher activity at lower concentrations (1 ng/ml) than IGF-I itself (Fig. 5B).

The potential biological significance of these results was further suggested by the demonstration that Clone 9 cells secrete IGF-I but not IGF-II into surrounding medium (Figs 6 A and B). Western blot analysis of concentrated medium clearly indicated a protein reactive with polyclonal anti-IGF-I antiserum (Fig. 6A, lane 4). Although IGF-II cross-reacts to a certain degree with the anti-IGF-I antiserum (lane 5), medium conditioned by Clone 9 cells does not react with anti-IGF-II antisera (Fig. 6B). We conclude that Clone 9 cells secrete IGF-I but not IGF-II into serum free medium. Clearly, therefore, the potential exists for locally produced IGF-I to act in an autocrine fashion at the IGF-I receptor (which we have shown to be functional in this cell line; Figs 3–5).

DISCUSSION

There are only limited reports of IGFBP-2 interaction with cell membranes or components of the extra-cellular matrix. By a process of *in vitro* autoradiography with ^{125}I-IGF-I, and immunohistochemistry with an anti-IGFBP-2 specific polyclonal antiserum, Russo *et al.*[12] reported the presence of IGFBP-2 in the mitral cell layer of the rat olfactory bulb. These findings were confirmed by the subsequent immuno precipitation of cross-linked ^{125}I-IGF-I–IGFBP complexes from solubilized olfactory bulb membranes with polyclonal anti-IGFBP-2 antisera. Despite the fact that IGFBP-2 contains a consensus RGD integrin binding sequence, IGFBP-2 association with

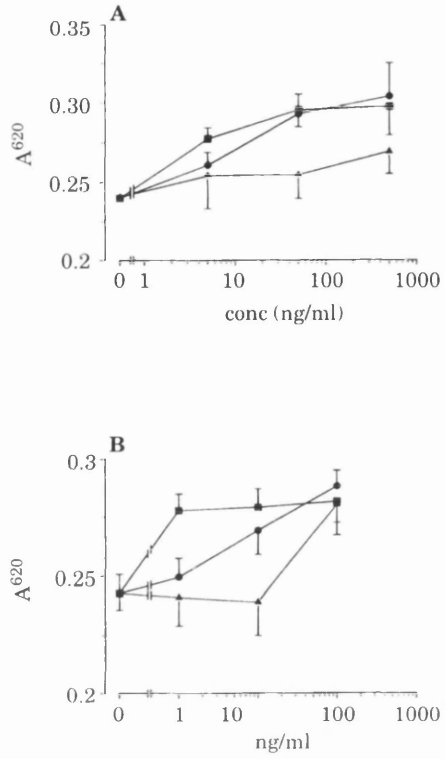

Fig. 5 An MTT metabolic/ proliferation assay following treatment of subconfluent cultures of Clone 9 cells. **(A)** Cultures were treated with IGF-I (•), des IGF-I (■) or insulin (▲) at the indicated concentrations. After 24 h incubation, microtitre plates were processed for MTT assay as described in Materials and Methods. **(B)** Cells were treated with IGF-I (•), des IGF-I (■) or IGF-II (▲) at the indicated concentrations and processed for MTT assay as described in (A). For both **(A)** and **(B)** the number of data points at each hormone concentration = 8. Results are expressed as mean ± SE. These experiments were repeated at least three times. The results of the MTT assay for the two treatments IGF-I and desIGF-I, at concentrations 1 and 10 ng/ml (Fig. 5B), were analysed with MINITAB version 11.2 / using a two-way ANOVA (with replications). The interaction between the hormones and the concentrations was significant ($P = 0.007$), indicating that the difference between the two treatments is dependent on the concentration. We then performed independent samples *t*-tests to compare the effect of IGF-I and desIGF-I at each of the two concentrations. The metabolic/mitogenic effect of the two hormones on Clone 9 cells, as determined with the MTT assay, was significantly different at the concentration of 1 ng/ml ($P < 0.0001$), but not at 10 ng/ml ($P = 0.071$).

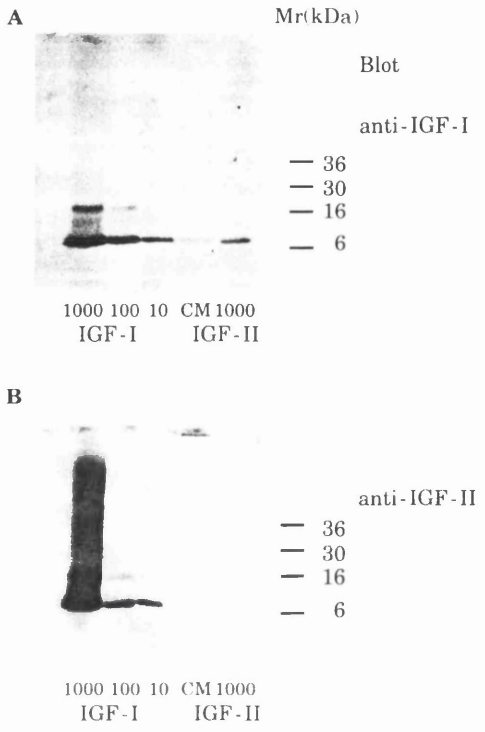

Fig. 6 Serum and protein free medium was conditioned by confluent monolayers of Clone 9 cells for a period of 24 h (A) IGF-I in concentrated conditioned medium (CM) was identified by Western blotting as described in Materials and Methods. Clearly evident is IGF-I derived from Clone 9 cell conditioned medium (lane 4) together with 1000 ng (lane 1), 100 ng (lane 2) and 10 ng (lane 3) of IGF-I standard. (B) In parallel blots, IGF-II was detected by probing with a polyclonal rabbit anti-human IGF-II antiserum. This experiment was repeated twice.

olfactory bulb membranes was not affected by the presence of an RGD – containing peptide. However, immunoprecipitation of BP-2 revealed the presence of a co-immunoprecipitating 200 kDa proteoglycan containing chondroitin sulphate. Indeed, in the same study it was reported that, *in vitro*, IGFBP-2 bound to the glycosaminoglycans- chondroitin-4 and -6 sulphate, keratan sulphate and heparin, leading to the suggestion that BP-2 may associate with these molecules on the cell membrane or in the extracellular matrix. In contrast, using an *in vitro* assay based on the binding of IGFBP-2 to heparin-Sepharose beads, it was demonstrated that IGFBP-2 bound to heparin and heparin sulphate, but not to chondroitin sulphate-A or -C or to dermatan sulphate[8]. Clearly, the exact components of the ECM/cell membranes responsible for the binding of IGFBP-2 require further elucidation. The effect of IGFBP-2 association with components of the cell membrane or extracellular matrix on the subsequent IGF-IGFBP interaction are also a subject of controversy. For example, whereas IGFBP-2 binding to chondroitin-6- sulphate has been reported to lower the affinity of the binding protein for IGF-I by a factor of 3[12], another study has reported that precomplexation of IGFBP-2 with either IGF-I or IGF-II is obligatory for the subsequent binding of the complex with heparin or the extracellular matrix components[8].

Clearly, the presence of cell surface – associated IGFBP-2 together with the IGF-I receptors on the cell membrane of Clone 9 cells has important consequences in the response of cells to local IGFs. In a similar vein, Reeve *et al.*[13] demonstrated the differential distribution

of membrane-associated IGFBP-2 between small cell and non-small cell lung carcinoma cultures (SMLC and NSCLC). SCLC cells, containing abundant membrane associated IGFBP-2 responded less well to exogenous IGF-I in terms of stimulation of DNA synthesis than the NSCLC cultures, which contain equivalent levels of cell membrane IGF-I receptors but little membrane-associated IGFBP-2. The findings of such studies imply an inhibitory role for membrane-associated IGFBP-2 and confirm the weight of evidence currently available in the literature in relation to this particular binding protein (reviewed in ref. 25). Similarly, in our current study, we find that the use of the non-IGFBP-binding IGF-I analogue des (1–3) IGF-I shows a greater activity over IGF-I in both long-term (24 h) mitogenic assays (Fig. 5) and in short-term signalling experiments (Figs 3 and 4). In the longer-term mitogenic assay, it is clearly possible that secreted IGFBP-2 may accumulate in the conditioned medium, bathing the cells to an extent such that activity of exogenous IGF-I is compromized. Over the shorter course of IGF-I signalling experiments, however, it is possible that the predominant inhibitory action on IGF-I activity is affected by membrane (ECM)-associated IGFBP-2, although this clearly is an hypothesis which requires further investigation.

Although IGF-II is reported to have a two-fold lower affinity for the IGF-I receptor than the homologous IGF-I ligand, we believe the activity of IGF-II at the IGF-I receptor is further compromised in our experimental system by the reported higher affinity of BP-2 for IGF-II. In addition, reports on relative affinities of IGF-I and IGF-II for the IGF-I receptor are largely documented for homologous human systems. Whether this holds for the rat proteins remains to be fully substantiated.

The rat liver cell line used in this current study is derived from the liver of an adult Sprague–Dawley rat[26]. It is epithelial in character and has been demonstrated previously to secrete IGFBP-2 into serum free medium[20]. Other parameters of the IGF axis are poorly characterized in rat liver cell lines, therefore our observations of the presence of functional IGF-I receptors and the secretion of IGF-I (but not IGF-II) by Clone 9 cells are of some significance. This latter property is shared by primary cultures of rat hepatocytes, although it is generally believed that levels of IGF-I receptors in adult hepatocytes are low and are increased only under conditions of hepatic regeneration or during fetal growth.[27] In the context of IGFBP secretion, whereas Clone 9 cells secrete only (or predominantly) IGFBP-2, a spectrum of IGFBPs – including IGFBP-1, -2 and -4–are produced by adult rat hepatocytes in culture[28]. To our knowledge, there has been no report of association of any IGFBPs with the surface of hepatocytes. Our comparison of the components of the IGF axis in Clone 9 cells, and that published to date for primary cultures of hepatocytes, emphasize the epithelial rather than parenchymal nature of this cell line. This is confirmed by our proliferation studies (Fig. 5A), where insulin is much less active than either IGF-I or des (1–3) IGF-I in a 24-h MTT assay. Such a pattern of reactivity is characteristic of insulin acting through the IGF-I receptor, for which it displays a lower affinity than for the homologous insulin receptor. This is confirmed by the signalling experiments reported in Fig. 3, where IGF-I and des (1–3) IGF-I are able to stimulate the tyrosine phosphorylation of 180 kDa protein at doses of 100 ng/ml, but insulin at concentrations of up to 1000 ng/ml has little effect on the tyrosine phosphorylation of this protein. Our observations on the proliferative effect of IGF-II in Clone 9 cells cultures also imply that the action of this polypeptide is via the IGF-I receptor, confirming the route of action described by other groups for the mitogenic effect of this polypeptide in cultures of rat hepatocytes, although others have suggested divergent mechanisms for IGF-I and IGF-II mitogenesis[29,30].

Clearly it would be of interest to identify the component(s) in the membrane of Clone 9 cells responsible for interaction with BP-2. One problem associated with this is the probable saturation of BP-2 binding sites by endogenously-produced IGFBP-2. In an attempt to overcome this, we are creating Clone 9 cell lines which are stable transfectants with anti-sense constructs of rat IGFBP-2. As well as addressing the difficulties indicated above, such cell lines should help in confirming the significance of the autocrine secretion of IGF-I in the regulation of cell growth. It has been reported very recently that human embryonic kidney fibroblasts transfected with a mouse IGFBP-2 expression vector, and which subsequently express high levels of mIGFBP-2, have slower growth curves than non-transfected cells[31]. Additionally, and similarly to our findings, the same study reports an enhanced activity for an alternative non-IGFBP binding IGF-I analogue (long R3 IGF-I) in stimulating growth of IGFBP-2 transfected cells arguing for the action of IGFBP-2 as a negative regulator of cell growth. Further evidence in support of this hypothesis is provided by the observation that the rat intestinal epithelial cell line IEC-6 stably transfected with an anti-sense rat BP-2 expression construct displays increased growth kinetics and growth arrested maximum density compared to non-transfected cells[32]. In this study, the authors were unable to demonstrate an association of IGFBP-2 with cell membranes. However, their data confirmed earlier results which indicated that IGF-I analogues with reduced binding affinity for IGFBPs, stimulated cell growth more potently than native IGF-I in this cell line,[33] although the authors did not investigate the association of IGFBP(s) with cell membranes. Together with our current observations, these data support a role for IGFBP-2 as an autocrine regulator of cell growth.

To conclude, in relation to the IGF axis in the rat liver derived epithelial cell line Clone 9, we have demonstrated that:

(1) This cell line contains functional IGF-I receptors and responds to IGF-I and des (1–3) IGF-I acutely (2–10 min) by increasing the level of tyrosine phosphorylation of a ~180 kDa protein, and chronically (24 h) by stimulating mitogenesis as determined by MTT assay.
(2) IGFBP-2 is secreted into the medium of Clone 9 cells, and a proportion of the binding protein associates with the plasma membrane of these cells.
(3) Secreted and membrane-bound forms of IGFBP-2 may inhibit the activity of IGF-I, as evidenced from the greater potency of the non-IGFBP binding analogue des (1–3) IGF-I in both signalling and mitogenic assays.
(4) This may have physiological relevance in the context of IGF-I action in an autocrine sense, as we demonstrate that Clone 9 cells secrete IGF-I but not IGF-II into conditioned medium.

We believe that this cell line may represent a useful model to investigate further local regulation and action of components of the IGF axis.

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