Aspects of insulin-like growth factor binding proteins in cancer

Matei Mireuta
Department of Experimental Medicine, Faculty of Medicine
McGill University, Montréal, Québec, Canada
August 2012

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy (Ph.D.)

Table of contents

Abstract

The insulin-like growth factor (IGF) system is composed of two ligands (IGF-1 and IGF-2), two receptors (IGF-1R and IGF-2R) and six binding proteins (IGFBP-1 to -6). IGFs act as endocrine, paracrine and autocrine growth factors and stimulate cell growth, proliferation and metabolism. There is extensive evidence, both from in vitro and in vivo models as well as population studies, that IGF physiology is relevant to neoplasia. IGF-1R is the physiologic receptor for both ligands and its activation elicits a plethora of changes at the cellular level, such as activation of PI3K/AKT/mTOR and Ras/Raf/MAP kinase pathways. Given its role in the maintenance and promotion of neoplasia, the IGF system represents a potential target in the context of cancer therapy.

Classically, IGFBPs have been described as carrier proteins for IGFs in the blood and other fluids. They can regulate IGF bioavailability both positively through increases in ligand half-life as well as negatively through competition with the IGF-1R for ligand binding. In addition to their classical roles, there is evidence suggesting that IGFBPs can act independently of IGFs by poorly characterized mechanisms. Additionally, epidemiologic studies have correlated overexpression of certain IGFBPs, in particular IGFBP-2, with poor prognosis in various cancers.

Although the role of IGFBPs has been extensively studied in the context of both normal and malignant growth, this thesis describes several new aspects of IGFBPs in neoplasia. In the second chapter, we study the effect of the PI3K/AKT/mTOR cascade on IGFBP-2 gene expression in a breast cancer cell line in vitro. We demonstrate that activation of this pathway essentially leads to an Sp1-dependent increase in IGFBP-2 gene transcription. We further show that Sp-1 is phosphorylated upon PI3K/AKT/mTOR pathway activation and accumulates in the nucleus. In the third chapter, we study the effects of 2-deoxyglucose (2-DG) on IGF-1:IGFBP-3 complex formation. A recent publication suggested that 2-DG unexpectedly disrupted IGF-1:IGFBP-3 binding leading to increases in IGF-1R and AKT signaling in various cell lines. We show by three

different techniques that neither 2-DG nor glucose affect IGF-1:IGFBP-3 complex formation. We additionally show that the 2-DG effects observed are not consistent between cell lines and likely the result of changes in intracellular signaling. In the fourth chapter, we study the effects of a novel therapeutic antibody (BI836845) with high affinity for both IGF-1 and IGF-2. In mouse serum samples ex vivo, we show that the addition of BI836845 leads to a shift of IGF-1 from the IGFBPs to the antibody. In vivo, we demonstrate that BI836845 binds the vast majority of IGF-1. Finally, we demonstrate that BI836845 induces a decrease in IGFBP-3 and an increase in growth hormone levels in C57 BL/6 mice.

Résumé

L'ensemble du système de facteurs de croissance insulinomimétique (IGF) est composé de deux ligands (IGF-1 et IGF-2), de deux récepteurs (IGF- 1R et IGF-2R) et de six protéines de liaison (IGFBP-1 à 6). Les IGFs sont des hormones endocrines, paracrines et autocrines qui stimulent la croissance cellulaire, la prolifération et le métabolisme. Il existe un grand nombre d'études utilisant des approches épidémiologiques ou des modèles in vivo et in vitro qui démontrent l'importance des IGFs dans le contexte du cancer. Le IGF-1R est le récepteur physiologique des deux ligands et son activation mène à d'importants changements cellulaires tels que l'activation des voies de signalisation PI3K/AKT/mTOR et Ras/Raf/MAPK. Étant donné son rôle dans la promotion et dans la progression du cancer, le système des IGFs représente une cible potentielle pour le traitement du cancer.

De façon classique, les protéines de liaison IGFBP ont été décrites comme de simples porteurs d'IGFs dans le sang et autres fluides. Les IGFBPs peuvent modifier la biodisponibilité des IGFs de façon positive en augmentant leur demi-vie ou de façon négative due à leur compétition avec le IGF-1R pour la liaison. En plus de leur rôle classique, il est de plus en plus évident que ces protéines peuvent agir de manière indépendante, mais les mécanismes impliqués restent flous. Également, il existe des études épidémiologiques qui ont corrélé la surexpression de IGFBPs, en particulier IGFBP-2, avec un pronostic défavorable dans plusieurs formes de cancer.

Bien que le rôle des IGFBPs ait été largement étudié dans le contexte de la croissance normale et en néoplasie, la présente thèse révèle quelques nouveaux aspects de la physiologie des IGFBPs dans le contexte du cancer. En première partie, nous étudions l'effet de la voie de signalisation PI3K/AKT/mTOR sur l'expression du gène IGFBP-2 dans une lignée cellulaire de cancer du sein. Nous démontrons que l'activation de cette voie mène essentiellement à une

augmentation de la transcription de ce gène de manière dépendante au facteur de transcription Sp-1. De plus, nous établissons que Sp-1 est phosphorylé par l'activation de la voie PI3K/AKT/mTOR et s'accumule dans le noyau. En deuxième partie, nous étudions les effets de la molécule 2-deoxyglucose (2-DG) sur la liaison entre IGF-1 et IGFBP-3. Un récent article avait suggéré un effet inhibitoire de cette molécule sur la formation de complexes IGF -1 :IGFBP-3. Nous démontrons par trois méthodes différentes que 2-DG ou la molécule apparentée glucose n'ont aucun effet sur la liaison entre IGF-1 et IGFBP-3. De plus, nous démontrons que les effets cellulaires de 2-DG sur l'activation de la voie PI3K/AKT/mTOR observées par les auteurs de l'article en question ne sont pas universels et sont probablement le résultat de signaux intracellulaires. Finalement, en dernière partie, nous étudions les effets d'un nouvel anticorps thérapeutique nommé BI836845 qui possède une grande affinité pour IGF-1 et IGF-2. Dans des échantillons de sérum de souris ex vivo, nous démontrons que l'ajout de BI836845 déplace IGF-1 des complexes naturels contenant les IGFBPs vers des complexes contenant l'anticorps. In vivo, nous démontrons que BI836845 lie la grande majorité d'IGF-1. Nous démontrons aussi que l'anticorps mène à une baisse de la concentration de IGFBP-3 et à une hausse de la concentration de l'hormone de croissance chez des souris C57 BL/6.

Acknowledgements

I would like to thank a great number of individuals who have contributed to the realization of this project. First of all, I would like to thank my supervisor, Dr. Michael Pollak, for his outstanding support, enthusiasm and encouragements. He was always available for discussion and provided much help and valuable input throughout the course of the project. I would also like to thank past and present members of Dr. Pollak's lab for their help and discussion particularly: Marie-José Blouin, Eva Yunhua Zhao, Mahvash Zakikhani, Elena Birman, Haider Mashhedi, Takamitsu Inoue, Shiva Javeshghani, Melinda Barmash, Miguel Bazile, Nader Toban, Yuzhen Tao, Ye Wang, Ying Gang Tu, Zhen Li, Zsuzanna Nagy, Carly Dool, Esther Piura, and Rocky Shi. I would especially like to thank Lillianne Lui for the tremendous help with formatting of the thesis and of the published manuscripts.

I would also like to thank colleagues of the LDI for their support with technical aspects, particularly Marguerite Buchanan and Daphné Richer. I would also like to thank our collaborators in published and unpublished manuscripts. Special thanks to Dr. Adam and Dr. Baxter for valuable reagents and insights and to Dr. Mark Hancock for performing the SPR experiments.

I would also like to thank my immediate family: Ioana, Neculai, Marina and Askim for the support throughout the course of the project. Last, but not least, I would like to thank my friends from the LDI, members of the famous Coffee Time /Osoko business club : Eric Segal, Andrew Darnel, Haider Mashhedi, Takamitsu Inoue, David Novak, Ari Reiss, Miguel Bazile and Sami Samir.

Finally I would like to thank Dr. Cheri Deal and Dr. Richard Bertrand for the correction of this thesis, your comments and input will greatly improve this work.

Again, thanks to everyone!

Thesis Introduction

Insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) are peptide hormones similar in molecular structure to insulin that regulate a variety of cellular activities, including metabolism, proliferation, and growth. The effects of IGFs are regulated by a family of six homologous IGF binding proteins (IGFBPs) which were originally described as simple IGF carrier proteins in the circulation and other fluids. However, it has been hypothesized that IGFBPs are not mere chaperones and that they can have effects of their own and independently of IGFs.

Although the role of IGFBPs has been extensively studied, the objective of this thesis is to expose several new aspects of the function of IGFBPs, particularly in the context of neoplasia and the experimental work undertaken. The first chapter of the thesis will present a literature review of the most important aspects of IGF physiology, particularly in the context of neoplasia. The second chapter will present an aspect of the regulation of IGFBP-2 in breast cancer cells. Then the third chapter will illustrate a study of IGF-1:IGFBP-3 complex formation in the context of 2-deoxyglucose treatment. The fourth chapter considers the study of a novel therapeutic candidate drug and its effects on IGFBP physiology. The final part of the thesis consists of a general discussion of the results. The relevant references are located at the end of each chapter.

The thesis is presented in manuscript based format. The preface of each chapter provides respective logical connecting bridges. The results in chapter 2 and 3 are adapted from published first author articles and the results in chapter 4 are adapted from a submitted manuscript. Chapters 2, 3 and 4 represent original contributions to the existing body of knowledge.

Contribution of authors

Apart from Dr. Pollak and myself, the following individuals appear as co-authors on the presented manuscripts and their contributions are as follows.

Chapter 2:

-Dr. Andrew Darnel was a member of the Lady Davis Institute working under Dr. Tarek Bismar. He provided much help with both reagents and technical aspects of cloning.

Chapter 3:

-Dr. Mark Hancock is part of the Sheldon Biotechnology Center at McGill University. He performed the surface plasmon resonance experiments and provided valuable insights.

Chapter 4:

-Dr. Paul Adam works at Boehringer Ingelheim (Vienna, Austria) and provided the therapeutic antibody BI836845 as well as insightful comments and project discussion.

-Melinda Barmash is a summer student in Dr. Pollak's Lab and she helped with discussion and manipulations.

-Elena Birman is a member of Dr. Pollak's Lab and performed the in vivo animal manipulations.

List of important abbreviations

µg	microgram
µl	microliter
µM	micromolar
ºC	degree Celsius
2-DG	2-deoxy-D-glucose
ALS	acid-labile subunit
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ERK	extracellular signal-related kinase
FBS	fetal Bovine Serum
GH	growth hormone
GHRH	growth hormone-releasing hormone
Grb	growth factor receptor-bound protein
IGF-1	insulin-like growth factor 1
IGF-2	insulin-like growth factor 2
IGF-1R	insulin-like growth factor type 1 receptor
IGF-2R	insulin-like growth factor type 2 receptor
IGFBP	insulin-like growth factor binding protein
IGFBP-rP	insulin-like growth factor binding protein-related protein
IR	insulin receptor
IRS	insulin receptor substrate
K_d	dissociation constant
kDa	kilo Dalton
MAPK	mitogen activated protein kinase
mg	milligram
ml	milliliter
mM	millimolar
mRNA	messenger ribonucleic acid
MS	mass spectrometry
mTOR	mammalian target of rapamycin
nM	nanomolar
O-GlcNAc	O-linked-N-acetylglucosamine
PBS	phosphate buffered saline
PDK-1	phosphoinositide-dependent kinase 1
PI3K	phosphaditylinositol-3'-kinase
PKB	protein kinase B
PTEN	phosphatase and tensin homolog
Raf	Ras-activating factor
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

SFM	serum free media
SHC	Src homology containing
SPR	surface Plasmon resonance
SUMO	small ubiquitin-like modifier
VEGF	vascular endothelial growth factor

List of figures and tables

Chapter 1:

Chapter 2:

Chapter 3:

Chapter 4:

Chapter 1

Literature Review

Preface

The first chapter of this thesis serves as a concise introduction to the field of IGF physiology, particularly in the context of neoplasia. The first section will describe the IGFs and their receptors, the subsequent section will be devoted to IGF binding proteins and the last part of the chapter will briefly introduce the rationale and some clinically relevant strategies for targeting the IGF axis in neoplasia.

1.1 Overview of IGF physiology

The Insulin-like growth factor (IGF) system is composed of two peptide hormones (IGF-1 and IGF-2), two receptors (IGF-1R and IGF-2R) and a family of binding proteins (IGFBP-1 to -6). In mammals, growth hormone (GH) is secreted by the pituitary gland, notably following growth hormone releasing hormone (GHRH) action, and acts on the liver to stimulate secretion of both IGF-1 and IGFBP-3. In contrast to GHRH, somatostatin is a short peptide hormone that inhibits growth hormone release (1). A schematic overview of the IGF system is presented in figure 1.1.

In circulation, the ligands are almost completely (>90%) found in complexes with IGFBPs. In serum, IGFBP-3 is the major binding protein and represents ~70% of IGFBPs. Both IGFBP-3 and -5 form ternary complexes with IGFs and a third protein termed Acid-Labile Subunit (ALS) (2). At the level of target tissues, IGF-1 and IGF-2 bind to the type 1 IGF receptor (IGF-1R) and lead to the activation of downstream signaling cascades. The activation of IGF-1R is associated with increased survival, proliferation and, in the case of cancer, to increased metastatic potential of the target cells (1). In contrast, the type 2 IGF receptor (IGF-2R), which binds primarily IGF-2, lacks a tyrosine kinase domain and therefore acts mainly as a "sink" for IGF-2. Additionally, the IGF-2R is also known as mannose-6-phosphate receptor and is involved in binding and internalization of ligands containing phosphorylated mannose moieties (3).

In addition to the endocrine actions of IGF peptide hormones, most normal (and neoplastic) tissues have been shown to secrete IGFs to some extent suggesting that autocrine/paracrine actions play an integral role in overall IGF activity. This endocrine/paracrine aspect of IGFs represents an important difference compared to the structurally related hormone insulin, which acts only systemically, or with other peptide growth factors such as EGF, which act locally (1). Additionally, IGFBPs are also secreted by tissues other than the liver and a considerable body of evidence suggests that their function might be more complex than their classical role of simple carrier proteins for IGFs (2).

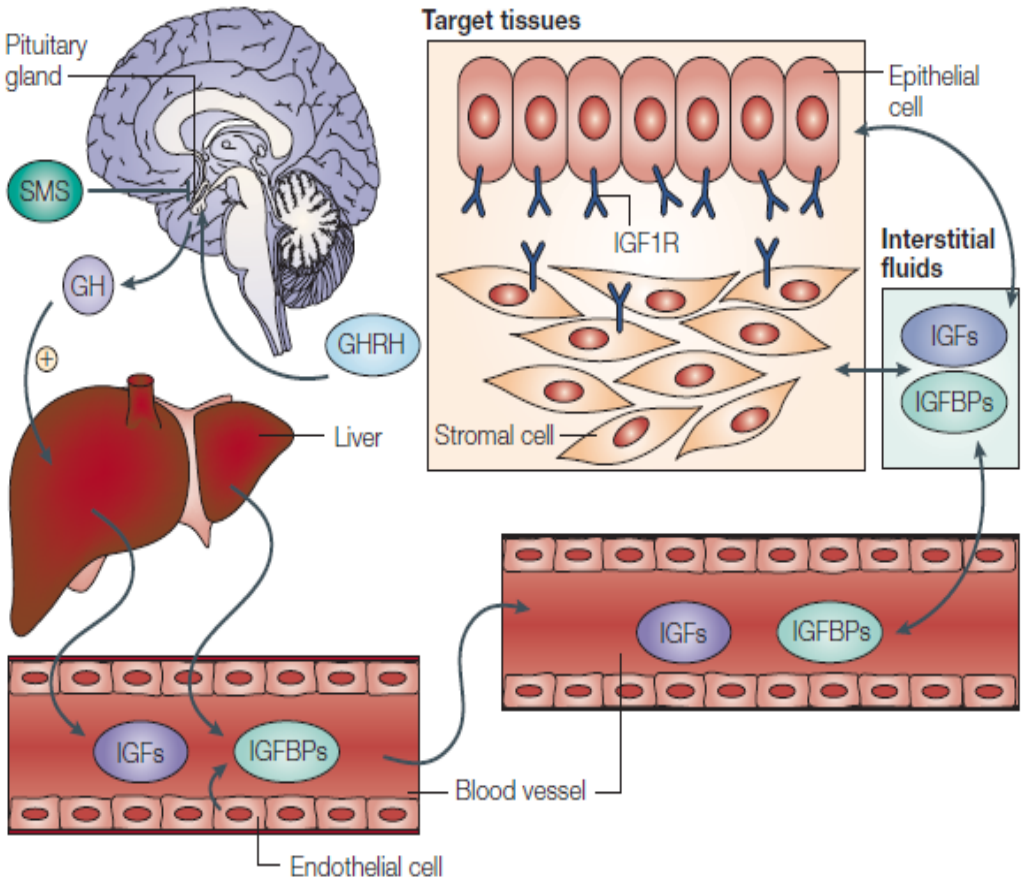


Figure 1.1: Schematic overview of the IGF system (Adapted from (1)). Growth hormone release is positively regulated by growth hormone releasing hormone (GHRH) and negatively by somatostatin (SMS) at the pituitary level. Growth hormone acts systemically on many organs and, in liver cells, it leads to increases in IGF-1 secretion. IGFs have characteristics of both endocrine hormones and autocrine/paracrine hormones as their secretion is ubiquitous.

1.2 Insulin-like growth factors

IGFs were initially known as somatomedins, in reference to a hypothesis postulated in the 1950s to explain the lack of activity of growth hormone (GH) on skeletal cartilage development in vitro. Salmon and Daughaday demonstrated that GH or serum from GH deficient rats did not affect sulfate incorporation by skeletal cartilage, but that serum from GH treated rats or normal rats increased sulfate incorporation (4). It was thought that a liver secreted factor present in

serum mediated most of the effects of GH and it was later named somatomedin (5).

At the same time, a different line of research was being conducted on the newly developed radioimmunoassay technique for the measurement of insulin. Froesch and colleagues discovered that the majority of insulin-like activity measured by this bioassay could not be suppressed by the addition of insulin antiserum (6). The group named this effect non-suppressible insulin-like activity (NSILA) and realized that it was present, in human serum, as a 100-150 kDa acid insoluble fraction and as a smaller acid insoluble fraction, which they named NSILA-P and NSILA-S respectively (7). Two insulin-like factors from NSILA-S were subsequently isolated (8) and named insulin-like growth factor 1 and 2 based on amino acid homology to proinsulin (9,10). In 1983, Klapper and colleagues demonstrated that IGF-1 and somatomedin-C were identical in amino acid sequence (11) and the name somatomedin was later abandoned (12).

It was soon recognized that IGFs were also expressed in many extrahepatic tissues (13,14). It was also increasingly evident that the pituitary GH was not the only regulator of expression of IGF-1 but that other factors such as nutritional status (15) were also involved. IGFs are part of an evolutionarily conserved system that impacts somatic growth, metabolism and ageing (16,17).

1.2.1 IGF-1

Insulin-like growth factor 1 (IGF-1) is a single chain peptide hormone of 70 amino acids containing 3 intrachain disulfide bonds. The IGF-1 gene is located in humans on the long arm of chromosome 12 (18) and spans more than 90 kbp of DNA. There are 6 exons (19) which give rise to several precursor mRNAs due to a combination of alternative splicing (20) and different transcription initiation sites (21). The different transcription initiation sites produce two classes of IGF-1

mRNAs, class I contains exon 1 as the leading exon, while the class II contains exon 2. Alternative splicing at the 3' end leads to the inclusion of exon 5 (IGF1 Eb) or exon 6 (IGF1 Ea) or both (IGF-1 Ec). The translated polypeptides therefore encode a variable amino terminal signal peptide and a variable carboxy terminal E-domain which are cleaved to yield mature IGF-1 (encoded by exons 3 and 4). The mature protein is in turn composed of 4 domains termed B,C,A and D in relationship to pro-insulin homology (22). The amino acid sequence is highly similar, and in some cases identical, among mammals.

As mentioned, IGF-1 is secreted by the liver as an endocrine hormone and by most tissues as an autocrine/paracrine factor. The regulation of IGF-1 is complex and tissue dependent. An important part of IGF-1 regulation is achieved through differential expression of the multiple mRNA and peptide precursors (23). There is evidence that class I IGF-1 mRNA are preferentially expressed in the liver and are more growth hormone dependent (22). Additionally, the length of the 5" untranslated region has been shown to affect translational efficiency of the precursors (24).

At the hepatic level, the regulation of IGF-1 is strongly influenced by growth hormone and nutritional status of the host. Additionally, several other factors such as castration (25), exercise status (26) and xenobiotic intake (27) have been implicated to affect IGF-1 serum or local levels.

IGF-1 has been shown to have extensive effects on tissues, organs and the organism as a whole. In mice, loss of IGF-1 leads to severe growth retardation (28) and depending on genetic background, to neonatal death (29). Interestingly, hepatic targeted IGF-1 loss-of-function leads to normal growth despite significantly decreased serum IGF-1 concentrations (30). Conversely, overexpression of IGF-1 in an organ specific fashion results mainly in improved function or hypertrophy of the targeted organs (31-33). In neoplasia, IGF-1 has been implicated in the progression and development of several tumors and antagonistic compounds represent an important field of cancer research.

1.2.2 IGF-2

Insulin-like growth factor 2 is also a small single chain peptide homologous both to proinsulin and IGF-1. The IGF-2 gene is located in humans on the short arm of chromosome 11, close to the insulin gene. The gene contains four promoters (P1-P4); transcription from P2, P3 and P4 occurs mainly in fetal and young tissues and is subject to imprinting (34-36). On the other hand, transcription from P1 occurs in adult liver and CNS in a biallelic fashion (36) but imprinting is preserved in other tissues (37). These transcripts generate propeptides that undergo posttranslational cleavage and the mature form is 67 amino acids in length and composed of similar B, A, C and D domains as IGF-1.

IGF-2 is expressed in a developmental and tissue specific fashion. In adult humans, the liver is arguably the main source of IGF-2, but growth hormone is not a major regulator. In knockout mouse models, loss of IGF-2 leads to growth retardation (38) and embryonic abnormalities (39). In transgenic models, IGF-2 overexpression lead to overgrowth, disproportionate organ sizes and considerable lethality (40,41). In neoplasia, overexpression of IGF-2 has been observed in several cancers and has been attributed to epigenetic aberrations such as loss of imprinting (42-45).

1.3 Insulin-like growth factor and related receptors

1.3.1 IGF-1 Receptor

The Insulin-like Growth Factor 1 Receptor (IGF-1R) gene is located on chromosome 15 and encodes a tyrosine kinase transmembrane receptor with 60% amino acid homology to the insulin receptor (IR). It consists of two extracellular alpha and two transmembrane beta domains disulfide linked in a tetrameric structure (46). Initially, the alpha and beta domains are translated as a single precursor of 1367 amino acids, which is folded through the aid of chaperones, glycosylated and trafficked to the Golgi. At this site, it is cleaved into the alpha and beta subunits which are subsequently disulfide linked (47). The full receptor is assembled at the cell surface. The alpha domain is involved in ligand binding, while the beta subunit is responsible for signal transduction (47).

IGF-1 and IGF-2 both exhibit considerable affinity for the IGF-1R and are considered the physiologic ligands (46,47). However, because of high homology between the IGF-1R and the IR, there is some degree of cross binding of ligands, although the involved affinities are much lower (48).

Expression of the IGF-1R is virtually ubiquitous and its regulation is influenced by many factors such as GH, estrogen and thyroid hormones (49). Interestingly, IGF-1 inhibits IGF-1R expression (50), in some contexts, and this represents a potential feed-back inhibition loop. Additionally, microRNAs (51) and tumor suppressor genes such as p53 and BRCA1 also downregulate IGF-1R expression (52).

In knockout mice, IGF-1R null homozygotes die shortly after birth and exhibit multiple organ abnormalities (53). In transgenic mice, tissue specific overexpression of IGF-1R leads to similar observations as with IGF-1 transgenic models (54,55). In neoplasia, the IGF-1R contributes to maintenance and progression of multiple cancers and is considered a potential target in the

treatment of malignancy (Section 1.6). IGF-1R associated signaling is shown in figure 1.2.

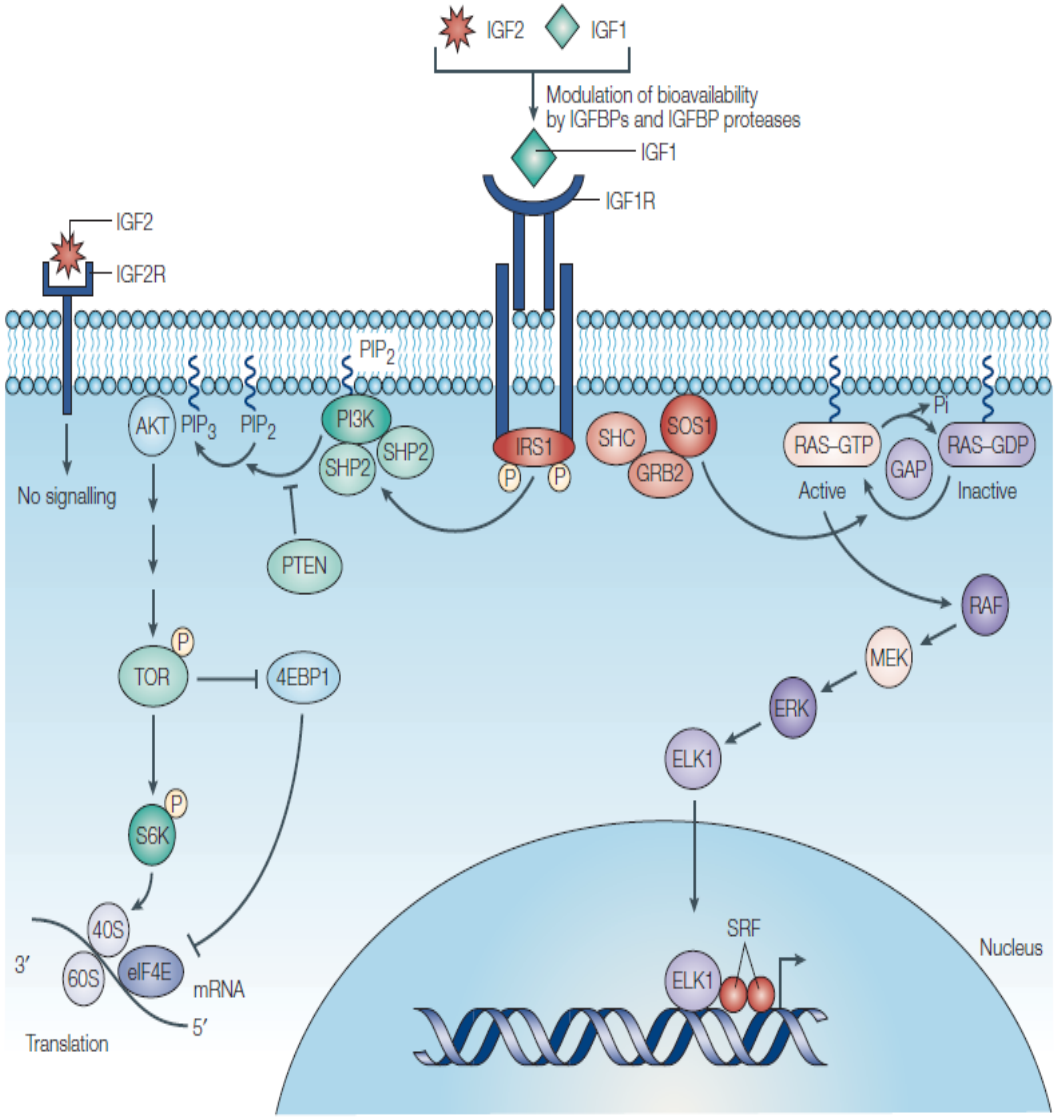


Figure 1.2: Schematic overview of the IGF-1R signaling pathways (Adapted from (1))

1.3.2 PI3K/AKT/mTOR Pathway

The binding of one IGF-1 or IGF-2 molecule to the alpha-domains of the IGF-1R leads to an ATP-dependent autophosphorylation reaction of a cluster in the intracellular domain of the beta-chain. This in turn results in an increase in intrinsic tyrosine kinase activity of the beta-domains and subsequently in the tyrosine phosphorylation of several docking proteins. These adaptor proteins then relay the signal to several downstream pathways. One such pathway is the PI3K/AKT/mTOR cascade. Phosphorylated tyrosine residues on the IGF-1R and insulin receptor substrate (IRS) docking proteins (56) serve to recruit the Phosphadityl Inositol 3" Kinase (PI3K) to the cell membrane (57). The PI3K enzyme is composed of two subunits; a regulatory p85 subunit responsible for phosphotyrosine binding through src homology 2 (SH2) domains and a p110 subunit catalyzing the conversion of membrane lipid phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$] to phosphatidylinositol-3,4,5-trisphosphate [$PI(3,4,5)P_3$] (57). The protein Akt, also known as protein kinase PKB, along with the phosphoinositide-dependent kinase 1 (PDK1) are recruited to the plasma membrane by binding to PIP3 through plekstrin homology (PH) domains (58). Akt is then fully activated by phosphorylation at two residues, namely T308 by PDK1 (59) and subsequently at S473 possibly by an mTOR complex (60) or DNA-dependent protein kinase (DNA-PK) (61). Once catalytically active, Akt elicits a plethora of cellular changes such as phosphorylation of transcription factors, inhibition of pro apoptotic proteins and activation of protein translation (62). The latter is achieved by activation of the mammalian target of rapamycin (mTOR) protein indirectly through inhibition of the tuberous sclerosis complex (TSC-1-TSC-2) (63). Activated mTOR then promotes the assembly of processed mRNA with the 40S ribosomal subunit as a result of direct phosphorylation of the translational repressor 4E-BP proteins (63). Additionally, mTOR activates S6 kinase (S6K) which in turn phosphorylates ribosomal protein S6 thereby enhancing translation, particularly of specific mRNAs containing a 5' oligopyrimidine stretch (64).

The PI3K/AKT/mTOR pathway signal can be negatively regulated by several mechanisms. For instance, an early checkpoint is the protein phosphatase PTEN which catalyzes the conversion of the AKT regulator PiP3 to PiP2 (65). For this reason, PTEN is considered a tumor suppressor gene and loss of function has been observed in various cancers (66). Furthermore, S6K promotes degradation of IRS-1 by phosphorylating the docking protein at several serine residues and thereby creating an effective negative feedback loop (67,68). Finally, other phosphatases negatively regulate signal transduction such as PH domain and Leucine rich repeat Protein Phosphatase (PHLPP) protein which dephosphorylates the S473 position of AKT (69).

1.3.3 The Ras/Raf/MEK/ERK pathway

The Ras/Raf/MEK/ERK pathway can also become activated following IGF-1R phosphorylation. Adaptor proteins Grb2 and Shc bind to the phosphorylated regions of the IGF-1R beta chains recruiting the son of sevenless (SOS) guanine exchange factor and the small G protein Ras (70). Active Ras recruits Raf to the plasma membrane by direct binding and Raf is then phosphorylated at several residues with Ser338 being among the most important for activation (70). The p21-activated protein kinase (PAK) has been shown to phosphorylate the Ser 338 position (70). Phosphorylation at other residues can be inhibitory, a notable cross-talk example is phosphorylation at the Ser 259 position by AKT (71). Once active, Raf kinase phosphorylates and activates MEK1 kinase which then activates extracellular regulated kinase (ERK) (70). The number of identified ERK substrates are in the hundreds, comprising transcription factors such as c-Jun and c-Myc, cytosolic kinases such as S6 kinase and apoptosis regulatory proteins to mention just a few (70). The end result of the Ras/Raf/Mek/ERK pathway activation is an increase in cellular proliferation, growth and an inhibition of apoptosis (70).

1.3.4 IGF-2 Receptor

The insulin-like growth factor receptor 2 (IGF-2R) also known as cation independent mannose-6 receptor is a monomeric transmembrane protein of approximately 300 kDa which can bind IGF-2 at much higher affinity than IGF-1 (3). The extracellular region contains domains homologous to the intracellular cation dependent mannose-6-phosphate receptor and can thus also bind mannose-6-phosphate moieties. Unlike the IGF-1R, its intracellular C-terminus is very short, does not possess intrinsic tyrosine kinase activity and hence does not elicit a signaling cascade (3). Contrary to IGF-2 which is maternally imprinted in humans (72,73), the IGF-2R is paternally imprinted, but only in mice (73). Upon IGF-2 binding, the IGF-2R is internalized in clathrin coated vesicles which fuse with early endosomes. IGF-2R is recycled back to the cell surface by the retromer complex while the ligand is degraded in lysosomes (3). For this reason, the prevalent consensus is that IGF-2R acts to regulate IGF-2 bioavailability.

In keeping with this idea, in knock-out studies, ablation of IGF-2R leads to overgrowth and early death of animals (74) a phenotype that can be reversed by IGF-2 gene inactivation (75).

1.3.5 IGF-1/IR Hybrid Receptors

As mentioned in section 1.3.1, the IGF-1R is assembled on the cell surface after pairing of two identical half-receptors. The same mechanism is true for the homologous insulin receptor and this system can lead to the formation of hybrid receptors composed of one half IGF-1R and one half IR receptors. Further complexity arises from the fact that the IR can be alternatively spliced and thus six different hybrid receptor combinations are possible. The six different receptor

species vary in their ligand affinities and this area remains a topic of ongoing research (76).

1.4 IGF Binding Proteins

The insulin-like growth factor binding proteins (IGFBPs) are a family of 6 (IGFBP-1 to -6) homologous proteins with IGF affinity in the low nanomolar range (2). Their structure is divided into three domains (N-terminal, central and C-terminal domain) of approximately equal sizes. The N and C-terminal domains are highly conserved and contain residues important for IGF binding. These regions are cysteine rich and contain several intracellular disulfide bridges. The central domain is not conserved among the 6 IGFBPs and contains sites for various posttranslational modifications such as glycosylation and phosphorylation. It also contains cleavage sites for various proteases and residues involved in ALS binding (IGFBP-3 and -5). The IGFBP genes are structurally similar; four relatively small exons arranged in a gene of several kb (as much as 30kb for IGFBP-2) (2). All IGFBP precursors contain signal peptides and the mature proteins are secreted extracellularly. IGFBPs have classically been defined as carrier proteins for IGFs and as IGF antagonists at the cellular level since they act to sequester IGFs away from the IGF-1R. However, a growing body of evidence suggests that their function is more complex, with reports showing IGF enhancing actions and even IGF-1R independent actions (2). A schematic illustration of IGFBPs is presented in figure 1.3.

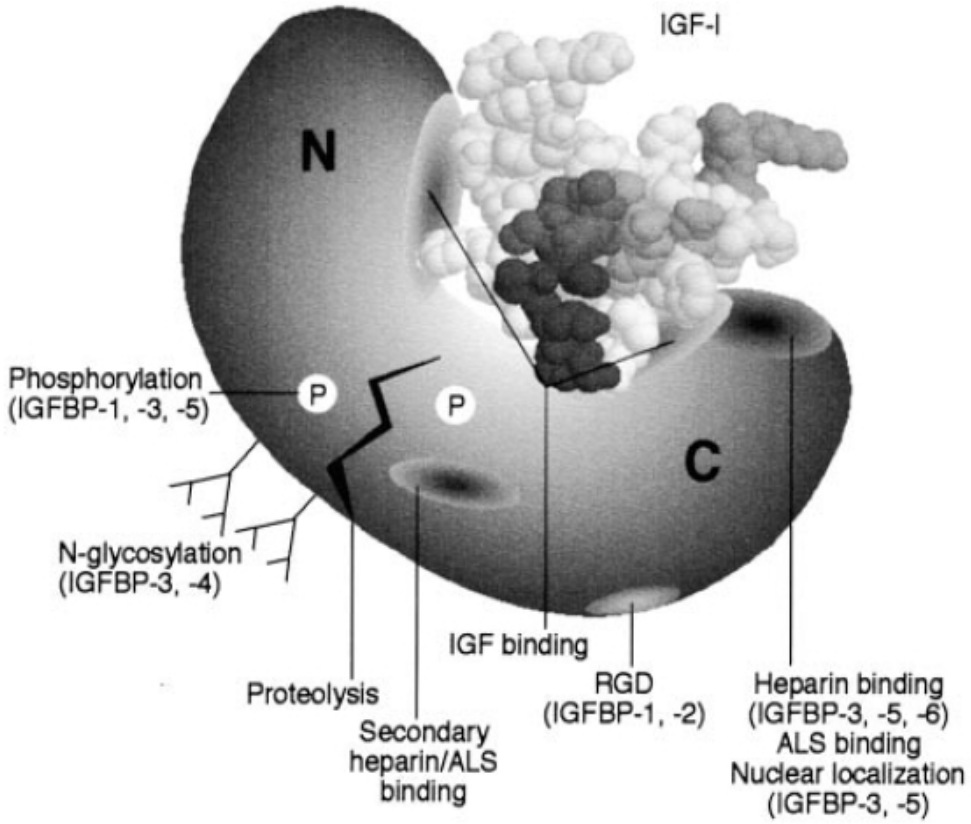


Figure 1.3: Schematic overview of IGFBPs (Adapted from (2))

1.4.1 IGFBP-1

IGFBP-1 was the first characterized family member (77). The gene is located contiguously to IGFBP-3 on chromosome 7 in humans and gives rise to a mature product of 234 amino acids. It is expressed in several tissues, most notably decidua, liver and kidney (78). The expression of IGFBP-1 is highly influenced by the metabolic state of the organism, with insulin being the most prominent negative regulator both in vitro and in vivo (79,80). It contains an RGD motif and has been shown to interact with cell surface integrins in various models in an IGF-1 independent fashion (81,82). Additionally, IGFBP-1 contains several phosphorylation sites which impact its affinity for IGF-1 (83). Dephosphorylated

IGFBP-1 has lower affinity and has been shown to enhance IGF-1 stimulated DNA synthesis, whereas the phosphorylated protein seems to promote IGF-1 actions (84). In vivo, transgenic mice experiments have shown that overexpression of rat IGFBP-1 leads to glucose intolerance and severe growth retardation (85), while overexpression of human IGFBP-1 in the liver has similar effects (86,87). In knock-out studies, no significant phenotypic changes were observed apart from a declined regeneration rate of the liver (88). These finding suggest that IGFBP-1 is mainly growth inhibitory in vivo.

1.4.2 IGFBP-2

IGFBP-2 was first isolated from rat liver (89). The gene is located on chromosome 2 near the IGFBP-5 gene in humans and gives rise to a mature product of 289 amino acids. It is expressed in several tissues and is the most abundant IGFBP in some areas such as cerebrospinal fluid (90). The expression of IGFBP-2 is complex and is influenced depending on tissue type by factors such as androgens (91), IGF-1 (92), glucocorticoids (93) and leptin (94). Similarly to IGFBP-1, it contains an integrin binding RGD domain which has been shown to mediate both IGF independent effects on motility (95) and integrin mediated signaling (96,97). In vitro, IGFBP-2 has been reported to behave controversially, either stimulating or inhibiting IGF actions depending on the cell lines and physiologic context (98). In vivo, transgenic mice overexpressing IGFBP-2 showed decreased weight gain consistent with a growth inhibitory role of IGFBP-2 (99). Conversely, mice with targeted deletions of IGFBP-2 had no significant phenotypic changes (100) but did display upregulation of other IGFBPs (101), a finding that is consistent with the idea of redundancy in the IGFBP family. In the context of neoplasia, overexpression of IGFBP-2 has been detected in several cancers (as discussed in the introduction to Chapter 2). Whether IGFBP-2

contributes to or merely is correlated with malignant transformation is still under investigation.

1.4.3 IGFBP-3

IGFBP-3 is the most abundant IGFBP in serum, present at a level 10-fold higher than other IGFBPs (2). It is (like IGFBP-5 (102)) capable of forming ternary complexes with IGFs and another protein termed Acid-labile Subunit (ALS). The mature core protein is composed of 264 amino acids but unlike other IGFBPs, it can be heavily N-glycosylated at three sites in the central domain (2). The C-terminal domain contains a heparin binding domain and has been shown to interact with various ligands at the cell surface (103). Additionally, the COOH terminal contains a nuclear localization sequence (104) and IGFBP-3 interaction with RXRα has been demonstrated (105). There are three putative membrane receptors specific for IGFBP-3: 1) the TGF-β type V receptor (106) also known as lipoprotein-related protein-1 (LRP-1)/α2M receptor (107) 2) a binding partner described years ago in Hs578T breast cancer cells (108) and 3) a recently cloned cell death receptor (possibly the same as in (108)) and termed IGFBP-3R (109). In addition, IGFBP-3 has also been shown to translocate across the cytosolic membrane by binding transferrin and caveolin-1, possibly involving a metal binding domain located at the C-terminus (110). Additionally, free IGFBP-3 was shown to inhibit DNA synthesis in a lung epithelial cell line, an effect that was abrogated by coincubation with IGF-1 (111). Despite the existing evidence, the IGFBP-3 putative receptors are not universally accepted as functional.

The hepatic regulation of IGFBP-3 transcription is influenced by growth hormone (112) which also results in a parallel upregulation of IGF-1. In addition to GH, TNF-alpha and Vitamin D, among many other compounds, were also reported to increase IGFBP-3 expression in cell lines and in rodents (113). Vitamin D and retinoids can be used as part of cancer therapy and have been shown to

decrease cellular proliferation in many instances (114,115). However, the contribution of elevated IGFBP-3 secretion to the observed anti-proliferative effects remains to be elucidated. On the other hand, several factors, such as androgens and EGF, have been reported to negatively regulate IGFBP-3 expression (113). Interestingly, the tumor suppressor p53 was shown to induce IGFBP-3 gene transcription through consensus sites located within the first intron of the gene (116). Therefore, factors affecting p53 activity such as irradiation or hypoxia can impact IGFBP-3 mRNA levels as well.

The actions of IGFBP-3 are mostly classified as growth inhibitory, although studies reporting increased proliferation in several experimental systems exist (117). It was proposed that IGFBP-3 can lead to IGF dependent growth stimulation by binding to cell membrane components and thereby leading to a concentration of IGFs in close proximity of the IGF-1R (2). In contrast, the mechanism invoked for growth inhibition is based on sequestration of IGFs as ligand affinity for IGFBP-3 is reportedly higher than for the IGF-1R (2).

On the other hand, certain IGFBP-3 actions that are IGF independent have been reported. In many cell line models, overexpression of IGFBP-3 or direct treatment with recombinant protein was reported to lead to decreased proliferation, increased DNA fragmentation and apoptosis (118-120). There are several mechanisms proposed for such observations. For example, as was demonstrated in a chondrocyte cell line, IGFBP-3 treatment can lead to the increase of pro-apoptotic STAT-1 nuclear localization, phosphorylation as well as mRNA levels (121). From another point of view, IGFBP-3 nuclear translocation and binding to RXRα was proposed to result in the mitochondrial translocation of the RXRα pro-apoptotic binding partner Nur77 (122). Although increased levels of mitochondrial Nur77 were observed in prostate xenografts following treatment with recombinant IGFBP-3 (123), other studies in breast cancer cell lines indicate that cell or nuclear membrane translocation of IGFBP-3 are not required for the induction of apoptosis (124). Finally, observations in breast cancer cell lines that IGFBP-3 can revert IGF-1 induced IGF-1R and IRS-1 phosphorylation lead to the

proposition that IGFBP-3 can induce the activity of a cellular phosphotyrosine phosphatase (125).

In knock-out studies, loss of IGFBP-3 had no effect on animal weights, but mice with triple deletions of IGFBP-3,-4 and -5 showed significant growth retardation and decreases in total IGF-1 serum levels (126). Conversely, overexpression of IGFBP-3 in transgenic mice lead to increases in heart and liver size in one study (127) and blocked mammary gland involution in another (128).

1.4.4 IGFBP-4

The IGFBP-4 gene is located in humans on chromosome 17. IGFBP-4 is the smallest IGFBP, contains 237 amino acids and, like IGFBP-3, it is subject to N-glycosylation in the central domain, albeit only at a single site (2). Contrary to other IGFBPs, the central domain of IGFBP-4 contains two cysteine residues which form and intradomain disulfide bond (2). The main source of IGFBP-4 in the blood is the liver, but its presence has been reported in most biological fluids and in many tissues (129). The hepatic regulation of IGFBP-4 is influenced by Vitamin D and Tri-iodothyronine and regulation in cell line models was reported to be influenced by multiple agents such as retinoic acid, estrogen and IGF-1 (129). The vast majority of studies on the effect of IGFBP-4 on cell growth in vitro have reported results consistent with IGF-dependent growth inhibition. In vivo, prostate cancer xenografts overexpressing IGFBP-4 displayed a reduction in tumor formation (130). In mouse mammary carcinoma cells, overexpression of wild-type IGFBP-4 induced no change in tumor development, but a protease resistant isoform exhibited a reduction in tumor growth (131). In transgenic mice, overexpression of IGFBP-4 induced decreased growth of thymus and a smaller weight of smooth muscle rich tissues (132). In IGFBP-4 knock-out studies, mice exhibited a small reduction of weight with no other significant changes (126).

1.4.5 IGFBP-5

IGFBP-5 contains 252 amino acids and, like IGFBP-3, contains a heparin binding domain in the C-terminal region (2). Like IGFBP-3, it exist in a trimeric complex with IGFs and ALS (102). It also contains phosphorylation sites and O-glycosylation sites. IGFBP-5 was also shown to be translocated to the nucleus, a process mediated mainly by importin-beta (104). IGFBP-5 can bind to a variety of molecules such as glycosaminoglycans, thrombospondin, vitronectin and osteopontin (2). In osteoblasts, IGFBP-5 was shown to interact with a cell membrane protein of about 420 kDa which was designated as a putative receptor (133). From yeast two-hybrid studies, there is evidence that IGFBP-5 has intracellular binding partners as well, in particular the transcription activator four-and-a-half LIM protein 2 (134) and the Ras association domain family member 1 gene product RASSF1C (135). The regulation of IGFBP-5 in vivo is under the influence mostly of IGF-1 and growth hormone in some instances (136). In vitro studies have reported several positive regulators of expression such as IGF-2 and insulin and some negative regulators such as dexamethasone and TSH (136). The actions of IGFBP-5 on cancer cell line models are reported, as is the case with IGFBPs in general, as either inhibiting or potentiating the actions of IGFs or as acting independently (136). In a xenograft study with breast cancer cell lines, IGFBP-5 overexpression was shown to inhibit tumor growth and to lead to increased apoptosis (137). In similar study with prostate cancer cells, the overexpression of IGFBP-5 lead to progression of the tumor to androgen independence (138). In transgenic mice, ubiquitous overexpression of IGFBP-5 lead to increases in neonatal mortality and whole body growth retardation (139). In knock-out studies, animals lacking IGFBP-5 expression were indistinguishable from controls with respect to growth and metabolism (126).

1.4.6 IGFBP-6

IGFBP-6 contains 213 amino acids and is located on chromosome 13 in man. Its N-terminal domain contains 2 less cysteines than other IGFBPs leading to the comparative lack of one intradomain disulfide bond (2). IGFBP-1 through -5 have almost equal affinity for IGF-1 or IGF-2, but IGFBP-6 has an approximately 50 times higher preference for IGF-2 (140). Similarly to IGFBP-5, IGFBP-6 also contains O-glycosylation sites (2). In vitro, IGFBP-6 regulation was influenced by differentiating agents such as retinoic acid (140). Actions of IGFBP-6 are mainly inhibitory in vitro and seem to be specific to IGF-2 due to the higher affinity for this ligand (140). In xenograft studies, overexpression of IGFBP-6 resulted in decreased growth of neuroblastoma (141) and nasopharyngeal carcinoma xenografts (142). In transgenic mouse studies, overexpression of IGFBP-6 under the glial fibrillary acidic protein lead to reduced body and cerebellum weights and impairments of the reproductive system (143). In knock-out studies, lack of IGFBP-6 lead to no significant difference in growth and development (144).

1.4.7 IGFBP proteases

Apart from the regulation of expression levels, IGFBP activity can be further impacted by a variety of proteases (2). A first account of IGFBP proteolysis was described about 20 years ago in human serum during pregnancy (145). Subsequent studies reported additional instances of IGFBP proteolysis and the first protease was identified as pregnancy associated plasma protein A (PAPP-A) and shown to cleave IGFBP-4 in an IGF dependent fashion (146). Since then, multiple proteases have been reported to modulated IGFBP function such as plasmin, thrombin, matrix metalloproteinase 1 through 3 (MMP1 -3), prostate specific antigen (PSA) and cathepsin L (2). The resulting fragments generally

have lower affinity for IGFs and therefore it is thought that IGFBP proteolysis is a mechanism that regulates IGF bioavailability. Additionally, this mechanism might also regulate IGF independent effects of IGFBPs although fragments are reported to have biological actions of their own (147).

1.4.8 ALS

Acid-labile subunit (ALS) is an ~85 kDa glycoprotein expressed in liver which is also regulated by growth hormone (148,149). It has limited affinity for free IGFs or IGFBPs and binds only binary complexed IGFBP-3 or IGFBP-5 to form 150 kDa ternary complexes which contain the vast majority of serum IGFs (150).

It was shown that ALS prolongs the half-life of ligands to about 12-15 hours as compared to minutes when in the free form or 1 hour when in the binary form (151). ALS knock-out mice exhibited some decrease in growth, but serum levels of both IGF-1 and IGFBP-3 were significantly reduced despite normal liver secretion, indicating that ALS is crucial for the maintenance of an active reservoir of ligand in the blood (152). Additionally, ALS functions to maintain IGFs in the circulation since ternary complexes have been shown to cross the capillary barrier less readily than binary forms (153).

1.4.9 IGFBPrPs

IGFBPs are members of a larger superfamily that also includes IGFBP related proteins (IGFBPrPs). IGFBPrPs are proteins that share significant homology to IGFBPs at the N terminal domain (154). These proteins were cloned independently and in different contexts and are therefore known under several acronyms. IGFBPrP-1 (Mac 25/TAF) was previously known as IGFBP-7 (155)

since it exhibits some, albeit limited affinity for IGFs (about 10 times lower than “classical” IGFBPs), but binds insulin with considerable affinity (156). In vitro and in vivo studies suggest that IGFBPrP1 acts as a tumor suppressor in several cancers (157-159). There are many other IGFBPrPs described in the literature, mostly members of the CCN protein family such as connective tissue growth factor (CGTF/IGFBPrP2) and nephroblastoma overexpressed H (NovH, IGFBPrP3) (154). These proteins may impact both normal and malignant cellular growth, but their effect on IGF physiology is less well characterized than for “classic” IGFBPs.

1.5 IGF physiology and cancer

In general, IGF-1R activation leads to decreased differentiation and increased proliferation of target cells, with few exceptions, notably in the process of myogenesis where IGF-1 leads to increased differentiation of myoblasts (160). Therefore, relevance of IGFs and the IGF-1R to carcinogenesis and to the progression of neoplasia is highly plausible, given that cancer is a disease related to control of cellular proliferation and differentiation, two processes that are normally regulated by IGF signaling. An early account of the role of the IGF-1 receptor in cellular transformation was made two decades ago when it was recognized that mouse embryonic fibroblasts from IGF-1R null mice cannot be transformed by a variety of oncogenes (159,161). Upon reintroduction of the IGF-1R gene, these cells regain susceptibility to transformation at similar levels to normal fibroblasts (159). The results were then extended to other systems with similar observations (162)).

In a more recent study, transgenic mice overexpressing a constitutively active IGF-1R isoform developed adenocarcinomas of the mammary and salivary glands (163). Similarly, transgenic mice overexpressing IGF-1 in basal epithelial cells of prostate showed spontaneous tumor formation in another study (164).

These and subsequent examples show that abnormalities of ligands or IGF-1R can contribute to neoplasia.

In a glioblastoma xenograft model, rats injected with a stably knocked down IGF-1R cell line did not develop tumors whereas control animals exhibited severe tumor burden (165). In a breast cancer xenograft model, MCF-7 cells injected in lit/lit mice ("little" mice), which are characterized by decreased growth hormone and IGF-1 levels, exhibited substantially reduced growth rates relative to control mice (166). Consistently, in different study, DU-145 cells were injected subcutaneously into nude mice and once tumors were visible, animals were either injected with PBS or IGF-1R antisense oligonucleotides (ASO). In ASO treated mice, the tumors regressed and were either absent or visible as small nodules after three additional months (162).

In vitro, IGF-1 is a mitogen in virtually all models, including glioblastoma, melanoma, breast, lung and prostate cancer cells (46). In these systems, IGF-1 was shown to confer strong protection from apoptosis inducing agents such as chemotherapy and radiation (46). In an in vivo study, p53 deficient mice were administered a bladder carcinogen which induced preneoplastic lesions and the effect of calorie restriction was studied. The calorie restriction lead to decreases in serum IGF-1 and to increased tumor apoptosis rates and decreased proliferation. Interestingly, this effect was attenuated with injections of recombinant IGF-1 (167). This provides evidence that the well-known inhibitory effects of calorie restriction on rodent carcinogenesis are mediated at least in part by a reduction in IGF-1 levels.

IGF-1R signaling has also been implicated in angiogenesis and metastasis. In vitro, IGF-1 was shown to be a potent upregulator of vascular endothelial growth factor (VEGF) in colon, bone and endometrial cancer cells (168-170). In vivo, a xenograft study of pancreatic cells expressing a dominant negative form of the IGF-1R receptor shows that disruption of IGF-1R signaling leads to decreased blood vessel density, decreased tumor weight and increased apoptosis (171). In

a breast cancer xenograft study, it was shown that IGF-1 induces increased tumor angiogenesis that can be attenuated by biphosphonates (172).

From the point of view of invasion/metastasis, the metastatic potential of murine carcinoma cells H-59 transfected with IGF-1R antisense was abrogated in a syngeneic model, whereas control H-59 cells lead to multiple liver and lung metastases (173). Moreover, human metastatic breast cancer cells MDA-MB-435 transfected with a dominant negative IGF-1R mutant lead to no difference in primary tumor growth when implanted in the mammary fat pad, but lead to significant decreases in secondary site metastases when compared to controls (174). Similarly, mouse lung cancer cells transfected with IGF-1R dominant negative mutant lead to decreased colonization, particularly of liver, over control mock transfected cells (175).

Additional evidence of the importance of the IGF-1/IGF-1R system in neoplasia comes from epidemiologic studies. It was shown that higher circulating levels of IGF-1 are correlated with increased cancer risk of breast (176), colon (177) and prostate (178). An interesting study monitored Ecuadorian subjects with decreased growth hormone and IGF-1 levels due to growth hormone receptor mutations. The study showed that these subjects had a lower cancer and diabetes incidence than control subjects over a 22 year period, although quantitative estimates of risk reduction were limited by sample size (179).

1.6 Targeting the IGF axis in cancer therapy

The body of evidence for the role of IGFs and IGF-1R in carcinogenesis and progression of neoplasia suggests that the IGF axis deserves investigation as a target for cancer therapy. There are several methods available to target IGF-1R signaling (figure 1.4). Over the past decade many IGF-targeting candidate drugs have been evaluated in pre-clinical models and some have been studied in

clinical trials. These consist of an inhibition of IGF-1R signaling through either the use of 1) receptor specific antibodies and 2) receptor tyrosine kinase inhibitors or 3) inhibitors of ligand bioactivity.

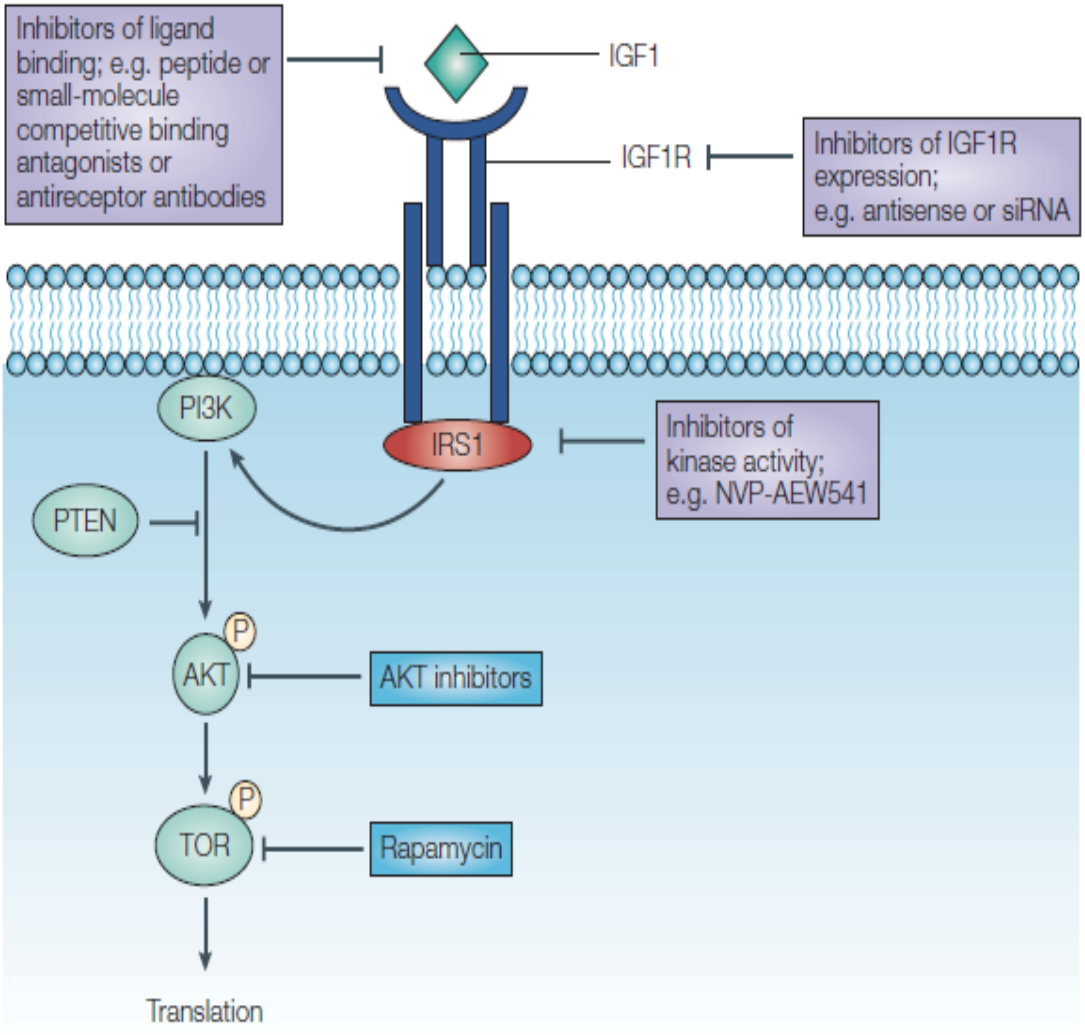


Figure 1.4: Schematic overview of IGF-1R targeting strategies (Adapted from (1)).

1.6.1 IGF-1R specific antibodies

There is evidence, for example from the development of trastuzumab (an antibody against the Her2/neu receptor), that growth factor receptor blockade can be beneficial in the treatment of cancer (180). There is additional evidence from in vivo experimental models which raises the possibility that IGF-1R receptor targeting may represent a useful strategy in cancer treatment (section 1.5). In the case IGF-1R specific antibodies, several agents have entered clinical trials over the past decade. The most extensive data available are from trials using Figitumumab (CP-751871, Pfizer). Figitumumab is a fully human antibody of subclass IgG2 with a half-life of 20 days (181). As a single agent, it appeared useful in the treatment of Ewing sarcoma in early clinical trials and in general exhibited low toxicity (182). Although most phase I/II studies suggested low toxicity, phase III trials of non-small cell lung cancer (NSCLC), where it was used in combinations, were halted due to disappointing effects and some toxicity, including hyperglycemia (183). Follow-up investigations have shown that the use of this antibody may cause substantial increases in insulin and IGF-1, possibly through compensatory growth hormone secretion (184). There is speculation that hyperinsulinemia may have attenuated the effects of IGF-1R blockade in tumors that expressed insulin receptors. Also, it must be recognized that IGF-1R activation is not hypothesized to be required for all cancers and future studies are designed to be restricted to IGF-1R dependent cancers, much as trastuzumab therapy is restricted to Her2/neu dependent cancers, rather than to unselected patients.

Other IGF-1R specific antibodies under study include AmG479 (Amgen), AvE1642 (Sanofi-Aventis) R1507 (Roche). These agents are well tolerated as single agents and some have shown promising activity in initial studies (185,186). However, R1507 was recently evaluated in combination with erlotinib for the treatment of advanced stage NSCLC with disappointing results (187). Clinical trials with these agents are ongoing and it was suggested that further

screening and selection of patients based on individual biomarkers might improve results (187). As mentioned, a notable effect of IGF1R specific antibodies is the elevation of total serum GH (and subsequently IGF-1) due to the disruption of the feed-back inhibition of IGF-1 on the hypothalamus (figure 1.1). However, there is no evidence to suggest that the elevated ligand levels can overcome the receptor blockade (188).

1.6.2 IGF-1R receptor tyrosine kinase inhibitors (RTKI)

In general, RTKI can function by competing with ATP for the ATP-binding pocket of the receptor or by allosteric modulation. Several RTKIs for the IGF-1R have been developed in the past decade such as BMS-536924 (189), OSI-906 (190) and NDGA (191). The similarity of the IGF-1R to the insulin receptor implies that these agents may not only inhibit the IGF-1R, but also hybrid receptors and insulin receptors. This could represent a theoretic advantage, but there is the possibility of metabolic toxicity. Despite these concerns, hyperglycemia has not been observed in early clinical trials (192), a finding that may relate to different accumulation of these agents in various relevant organs (193).

1.6.3 Modulation of ligand levels/bioactivity

The clinical usefulness of bevacizumab, a VEGF specific antibody, demonstrates that ligand targeting strategies are clinically viable in cancer treatment (194). In the case of IGFs, an early attempt to reduce their levels was through the use of somatostatin analogues which were intended to reduce GH levels which in turn reduce total IGF-1 levels. These strategies were clinically unsuccessful (192) and other strategies such as IGF peptide analogs (195) have not been evaluated clinically. A newer approach to develop ligand specific antibodies has yielded two

promising candidates, MEDI-537 (Astra Zeneca (196)) and BI836845 (Boehringer Ingelheim (197)).

These monoclonal antibodies have shown impressive efficacy in experimental models and have recently entered clinical trials. In rodent models, BI836845 administration leads to large increases in total IGF-1 serum levels and this effect is hypothesized to originate either from similar host compensation as is observed with IGF-1R specific antibodies or from a longer ligand half-life due to antibody binding. The complete characterization of ligand specific antibody effects is the subject of ongoing clinical and experimental studies. An important point particular to antibodies that bind IGF-1 and IGF-2 is their potential to generate free IGFBPs in serum. As was discussed in section 1.4, IGFBPs can have IGF-independent effects and therefore could influence the antineoplastic activity of the therapeutic antibodies. Thus, patients treated with high affinity ligand specific antibodies may develop an unusual serum IGFBP profile and the consequences of this process are not currently characterized.

Other targeting strategies of interfering with the IGF axis, such as antisense oligonucleotide or siRNA treatments specific for the IGF-1R remain experimental at the moment.

1.7 Introduction to experimental work

There are many areas of ongoing investigation related to IGFBP physiology both in general and in the context of neoplasia. In my experimental work, I have focused on:

- The regulation of expression of IGFBP-2 in breast cancer cells.
- The hypothesis that 2-DG interferes with IGF-1:IGFBP-3 binding.
- The impact of a novel drug candidate, the anti-ligand antibody BI836845, on IGF-1:IGFBP-3 binding.

The results described in chapters 2, 3 and 4 represent original contributions to the current body of knowledge of IGFBP physiology.

Chapter References

1. Pollak MN, Schernhammer ES, Hankinson SE. Insulin-like growth factors and neoplasia. Nat Rev Cancer 2004;4:505-18.

2. Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev 2002;23:824-54.

3. El Shewy HM, Luttrell LM. Insulin-like growth factor-2/mannose-6 phosphate receptors. Vitam Horm 2009;80:667-97.

4. Salmon WD, Daughaday WH. A hormonally controlled serum factor which stimulated sulfate incorporation by cartilage *in vitro.* J Lab Clin Med 1957;49:825-36.

5. Daughaday WH, Hall K, Raben MS, Salmon WD, Jr., Brande JL, van d, et al. Somatomedin: proposed designation for sulphation factor. Nature 1972;235:107.

6. Froesch ER, Burgi H, Ramseier E, Bally P, Labhart A. Antibody suppressible and nonsuppressible insulin-like activities in human serum and their physiologic significance. J Clin Invest 1963;42:1816-34.

7. Burgi H, Muller WA, Humbel RE, Labhart A, Froesch ER. Non-suppressible insulin-like activity of human serum. I. Physicochemical properties, extraction and partial purification. Biochim Biophys Acta 1966;121:349-59.

8. Rinderknecht E, Humbel RE. Polypeptides with nonsuppressible insulin-like and cell-growth promoting activities in human serum: isolation, chemical characterization, and some biological properties of forms I and II. Proc Natl Acad Sci U S A 1976;73:2365-9.

9. Rinderknecht E, Humbel RE. The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. J Biol Chem 1978;253:2769-76.

10. Rinderknecht E, Humbel RE. Primary structure of human insulin-like growth factor II. FEBS Lett 1978;89:283-6.

11. Klapper DG, Svoboda ME, Van Wyk JJ. Sequence analysis of somatomedin-C: confirmation of identity with insulin-like growth factor I. Endocrinol 1983;112:2215-7.

12. Daughaday WH, Hall K, Salmon WD, Jr., Van den Brande JL, Van Wyk JJ. On the nomenclature of the somatomedins and insulin-like growth factors. J Clin Endocrinol Metab 1987;65:1075-6.

13. D'Ercole AJ, Applewhite GT, Underwood LE. Evidence that somatomedin is synthesized by multiple tissues in the fetus. Developmental Biology 1980;75:315-28.

14. D'Ercole AJ, Stiles AD, Underwood LE. Tissue concentrations of somatomedin C: further evidence for multiple sites synthesis and paracrine or autocrine mechanisms of action. Proc Natl Acad Sci USA 1984;81:935-9.

15. Clemmons DR, Underwood LE. Nutritional regulation of IGF-I and IGF binding proteins. Annu Rev Nutr 1991;11:393-412.

16. Barbieri M, Bonafe M, Franceschi C, Paolisso G. Insulin/IGF-I-signaling pathway: an evolutionarily conserved mechanism of longevity from yeast to humans. Am J Physiol Endocrinol Metab 2003;285:E1064-E1071.

17. Ohlsson C, Mohan S, Sjogren K, Tivesten A, Isgaard J, Isaksson O, et al. The role of liver-derived insulin-like growth factor-I. Endocr Rev 2009;30:494-535.

18. Hoppener JW, Pagter-Holthuizen P, Geurts van Kessel AH, Jansen M, Kittur SD, Antonarakis SE, et al. The human gene encoding insulin-like growth factor I is located on chromosome 12. Hum Genet 1985;69:157-60.

19. Kim SW, Lajara R, Rotwein P. Structure and function of a human insulin-like growth factor-I gene promoter. Mol Endocrinol 1991;5:1964-72.

20. Rotwein P. Structure, evolution, expression and regulation of insulin-like growth factors I and II. Growth Factors 1991;5:3-18.

21. Jansen E, Steenbergh PH, LeRoith D, Roberts CT, Sussenbach JS. Identification of multiple transcription start sites in the human insulin-like growth factor-I gene. Mol Cell Endo 1991;78:115-25.

22. Shavlakadze T, Winn N, Rosenthal N, Grounds MD. Reconciling data from transgenic mice that overexpress IGF-I specifically in skeletal muscle. Growth Horm IGF Res 2005;15:4-18.

23. Rotwein P, Bichell DP, Kikuchi K. Multifactorial regulation of IGF-I gene expression. Mol Reprod Dev 1993;35:358-63.

24. Yang H, Adamo ML, Koval AP, McGuinness MC, Ben Hur H, Yang Y, et al. Alternative leader sequences in insulin-like growth factor I mRNAs modulate translational efficiency and encode multiple signal peptides. Mol Endocrinol 1995;9:1380-95.

25. Ohlson N, Bergh A, Stattin P, Wikstrom P. Castration-induced epithelial cell death in human prostate tissue is related to locally reduced IGF-1 levels. Prostate 2007;67:32-40.

26. Jiang Y, Ma H, Su X, Chen J, Standard J, Lin D, et al. IGF-1 Mediates Exercise-Induced Phospholipid Alteration in the Murine Skin Tissues. J Nutr Food Sci 2012;S2:003-doi:10.4172/2155-9600.S2-003.

27. Scarth JP. Modulation of the growth hormone-insulin-like growth factor (GH-IGF) axis by pharmaceutical, nutraceutical and environmental xenobiotics: an

emerging role for xenobiotic-metabolizing enzymes and the transcription factors regulating their expression. A review. Xenobiotica 2006;36:119-218.

28. Liu JL, Grinberg A, Westphal H, Sauer B, Accili D, Karas M, et al. Insulin-like growth factor-I affects perinatal lethality and postnatal development in a gene dosage-dependent manner: manipulation using the Cre/loxP system in transgenic mice. Mol Endocrinol 1998;12:1452-62.

29. Braxton LP, Hollingshead P, Warburton C, Dowd M, Pitts-Meek S, Dalton D, et al. IGF-I is required for normal embryonic growth in mice. Genes Dev 1993;7:2609-17.

30. Yakar S, Liu JL, Stannard B, Butler A, Accili D, Sauer B, et al. Normal growth and development in the absence of hepatic insulin-like growth factor I. Proc Natl Acad Sci USA 1999;96:7324-9.

31. Musaro A, McCullagh K, Paul A, Houghton L, Dobrowolny G, Molinaro M, et al. Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. Nat Genet 2001;27:195-200.

32. Li Q, Wu S, Li SY, Lopez FL, Du M, Kajstura J, et al. Cardiac-specific overexpression of insulin-like growth factor 1 attenuates aging-associated cardiac diastolic contractile dysfunction and protein damage. Am J Physiol Heart Circ Physiol 2007;292:H1398-H1403.

33. Konno-Takahashi N, Takeuchi T, Shimizu T, Nishimatsu H, Fukuhara H, Kamijo T, et al. Engineered IGF-I expression induces glandular enlargement in the murine prostate. J Endocrinol 2003;177:389-98.

34. Ekstrom TJ, Cui H, Nystrom A, Rutanen EM, Ohlsson R. Monoallelic expression of IGF2 at the human fetal/maternal boundary. Mol Reprod Dev 1995;41:177-83.

35. Gray A, Tam AW, Dull TJ, Hayflick J, Pintar J, Cavenee WK, et al. Tissue-specific and developmentally regulated transcription of the insulin-like growth factor 2 gene. DNA 1987;6:283-95.

36. Vu TH, Hoffman AR. Promoter-specific imprinting of the human insulin-like growth factor-II gene. Nature 1994;371:714-7.

37. Ohlsson R, Hedborg F, Holmgren L, Walsh C, Ekstrom TJ. Overlapping patterns of IGF2 and H19 expression during human development: biallelic IGF2 expression correlates with a lack of H19 expression. Development 1994;120:361-8.

38. DeChiara TM, Efstratiadis A, Robertson EJ. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. Nature 1990;345:78-80.

39. Coan PM, Fowden AL, Constancia M, Ferguson-Smith AC, Burton GJ, Sibley CP. Disproportional effects of Igf2 knockout on placental morphology and diffusional exchange characteristics in the mouse. J Physiol 2008;586:5023-32.

40. Ward A, Bates P, Fisher R, Richardson L, Graham CF. Disproportionate growth in mice with Igf-2 transgenes. Proc Natl Acad Sci U S A 1994;91:10365-9.

41. Sun FL, Dean WL, Kelsey G, Allen ND, Reik W. Transactivation of Igf2 in a mouse model of Beckwith-Wiedemann syndrome. Nature 1997;389:809-15.

42. Rainier S, Johnson LA, Dobry CJ, Ping AJ, Grundy PE, Feinberg AP. Relaxation of imprinted genes in human cancer. Nature 1993;362:747-9.

43. Kaneda A, Feinberg AP. Loss of imprinting of IGF2: a common epigenetic modifier of intestinal tumor risk. Cancer Res 2005;65:11236-40.

44. Bhusari S, Yang B, Kueck J, Huang W, Jarrard DF. Insulin-like growth factor-2 (IGF2) loss of imprinting marks a field defect within human prostates containing cancer. Prostate 2011;71:1621-30.

45. Polychronakos C, Giannoukakis N, Deal CL. Imprinting of IGF2, insulin-dependent diabetes, immune function, and apoptosis: a hypothesis. Dev Genet 1995;17:253-62.

46. Yu H, Rohan T. Role of the insulin-like growth factor family in cancer development and progression. J Natl Cancer Inst 2000;92:1472-89.

47. Riedemann J, Macaulay VM. IGF1R signalling and its inhibition. Endocr Relat Cancer 2006;13 Suppl 1:S33-S43.

48. Steele-Perkins G, Turner J, Edman JC, Hari J, Pierce SB, Stover C, et al. Expression and characterization of a functional human insulin-like growth factor I receptor. J Biol Chem 1988;263:11486-92.

49. LeRoith D, Werner H, Beitner-Johnson D, Roberts C. Molecular and cellular aspects of the insulin-like growth factor I receptor. Endocr Rev 1995;16:143-63.

50. Hernandez-Sanchez C, Werner H, Roberts CT, Jr., Woo EJ, Hum DW, Rosenthal SM, et al. Differential regulation of insulin-like growth factor-I (IGF-I) receptor gene expression by IGF-I and basic fibroblastic growth factor. J Biol Chem 1997;272:4663-70.

51. Liang R, Khanna A, Muthusamy S, Li N, Sarojini H, Kopchick JJ, et al. Post-transcriptional regulation of IGF1R by key microRNAs in long-lived mutant mice. Aging Cell 2011;10:1080-8.

52. Werner H, Roberts CT, Jr. The IGFI receptor gene: a molecular target for disrupted transcription factors. Genes Chromosomes Cancer 2003;36:113-20.

53. Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A. Mice carrying null mutations of the genes encoding insulin-like growth factor (IGF-1) and type 1 IGF receptor (IGF1R). Cell 1993;75:59-72.

54. Huynh K, McMullen JR, Julius TL, Tan JW, Love JE, Cemerlang N, et al. Cardiac-specific IGF-1 receptor transgenic expression protects against cardiac

fibrosis and diastolic dysfunction in a mouse model of diabetic cardiomyopathy. Diabetes 2010;59:1512-20.

55. Imrie H, Viswambharan H, Sukumar P, Abbas A, Yuldasheva N, Gage M, et al. 109 Overexpression of endothelial insulin-like growth factor-1 receptors (IGF-1R): a novel role for IGF-1R in endothelial function and repair. Heart 2012;98:A62-doi:10.1136/heartjnl-2012-301877b.109.

56. White MF. The IRS-signalling system: a network of docking proteins that mediate insulin action. Mol Cell Biochem 1998;182:3-11.

57. Cantley LC. The phosphoinositide 3-kinase pathway. Science 2002;296:1655-7.

58. Franke TF, Kaplan DR, Cantley LC, Toker A. Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. Science 1997;275:665-8.

59. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, et al. Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J 1996;15:6541-51.

60. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 2005;307:1098-101.

61. Feng J, Park J, Cron P, Hess D, Hemmings BA. Identification of a PKB/Akt hydrophobic motif Ser-473 kinase as DNA-dependent protein kinase. J Biol Chem 2004;279:41189-96.

62. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell 2007;129:1261-74.

63. Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev 2004;18:1926-45.

64. Meyuhas O, Hornstein E. Translational control of TOP mRNAs. 2000;671-94.

65. Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem 1998;273:13375-8.

66. Salmena L, Carracedo A, Pandolfi PP. Tenets of PTEN tumor suppression. Cell 2008;133:403-14.

67. Um SH, D'Alessio D, Thomas G. Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. Cell Metab 2006;3:393-402.

68. Harrington LS, Findlay GM, Gray A, Tolkacheva T, Wigfield S, Rebholz H, et al. The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. J Cell Biol 2004;166:213-23.

69. Gao T, Furnari F, Newton AC. PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. Mol Cell 2005;18:13-24.

70. Pearson G, Robinson F, Beers GT, Xu BE, Karandikar M, Berman K, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 2001;22:153-83.

71. Reusch HP, Zimmermann S, Schaefer M, Paul M, Moelling K. Regulation of Raf by Akt controls growth and differentiation in vascular smooth muscle cells. J Biol Chem 2001;276:33630-7.

72. Giannoukakis N, Deal C, Paquette J, Goodyer CG, Polychronakos C. Parental genomic imprinting of the human IGF2 gene. Nature Genet 1993;4:98-101.

73. Kalscheuer VM, Mariman EC, Schepens MT, Rehder H, Ropers HH. The insulin-like growth factor type-2 receptor gene is imprinted in the mouse but not in humans. Nature 1993;5:74-7.

74. Lau MM, Stewart CE, Liu Z, Bhatt H, Rotwein P, Stewart CL. Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. Genes Dev 1994;8:2953-63.

75. Ludwig T, Eggenschwiler J, Fisher P, D'Ercole AJ, Davenport ML, Efstratiadis A. Mouse mutants lacking the type 2 IGF receptor (IGF2R) are rescued from perinatal lethality in Igf2 and Igf1r null backgrounds. Dev Biol 1996;177:517-35.

76. Belfiore A, Frasca F, Pandini G, Sciacca L, Vigneri R. Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. Endocr Rev 2009;30:586-623.

77. Lee Y, Hintz RL, James PM, Lee PDK, Shively JE, Powell DR. Insulin-like growth factor (IGF) binding protein complementary deoxyribonucleic ackd from human HEP G2 hepatoma cells: Predicted protein sequence suggests an IGF binding domain different from those of the IGF-I and IGF-II receptors. Mol Endocrinol 1988;2:404-11.

78. Lee PD, Giudice LC, Conover CA, Powell DR. Insulin-like growth factor binding protein-1: recent findings and new directions. Proc Soc Exp Biol Med 1997;216:319-57.

79. Frystyk J. Free insulin-like growth factors -- measurements and relationships to growth hormone secretion and glucose homeostasis. Growth Horm IGF Res 2004;14:337-75.

80. Unterman TG, Oehler DT, Murphy LJ, Lacson RG. Multihormonal regulation of insulin-like growth factor-binding protein-1 in rat H4IIE hepatoma cells: the dominant role of insulin. Endocrinol 1991;128:2693-701.

81. Gleeson LM, Chakraborty C, McKinnon T, Lala PK. Insulin-like growth factor-binding protein 1 stimulates human trophoblast migration by signaling through

alpha 5 beta 1 integrin via mitogen-activated protein Kinase pathway. J Clin Endocrinol Metab 2001;86:2484-93.

82. Perks CM, Newcomb PV, Norman MR, Holly JM. Effect of insulin-like growth factor binding protein-1 on integrin signalling and the induction of apoptosis in human breast cancer cells. J Mol Endocrinol 1999;22:141-50.

83. Jones JI, Busby WH, Jr., Wright G, Clemmons DR. Human IGFBP-1 is phosphorylated on 3 serine residues: effects of site-directed mutagenesis of the major phosphoserine. Growth Regul 1993;3:37-40.

84. Jones JI, D'Ercole AJ, Camacho-Hubner C, Clemmons DR. Phosphorylation of insulin-like growth factor (IGF)-binding protein 1 in cell culture and in vivo: effects on affinity for IGF-I. Proc Natl Acad Sci USA 1991;88:7481-5.

85. Rajkumar K, Barron D, Lewitt MS, Murphy LJ. Growth retardation and hyperglycemia in insulin-like growth factor binding protein-1 transgenic mice. Endocrinol 1995;136:4029-34.

86. Gay E, Seurin D, Babajko S, Doublier S, Cazillis M, Binoux M. Liver-specific expression of human insulin-like growth factor binding protein-1 in transgenic mice: repercussions on reproduction, ante- and perinatal mortality and postnatal growth. Endocrinol 1997;138:2937-47.

87. Ben Lagha N, Seurin D, Le Bouc Y, Binoux M, Berdal A, Menuelle P, et al. Insulin-like growth factor binding protein (IGFBP-1) involvement in intrauterine growth retardation: study on IGFBP-1 overexpressing transgenic mice. Endocrinol 2006;147:4730-7.

88. Leu JI, Crissey MA, Craig LE, Taub R. Impaired hepatocyte DNA synthetic response posthepatectomy in insulin-like growth factor binding protein 1-deficient mice with defects in C/EBP beta and mitogen-activated protein kinase/extracellular signal-regulated kinase regulation. Mol Cell Biol 2003;23:1251-9.

89. Mottola C, MacDonald RG, Brackett JL, Mole JE, Anderson JK, Czech MP. Purification and amino-terminal sequence of an insulin-like growth factor-binding protein secreted by rat liver BRL-3A cells. J Biol Chem 1986;261:11180-8.

90. Chesik D, De Keyser J, Wilczak N. Insulin-like growth factor binding protein-2 as a regulator of IGF actions in CNS: implications in multiple sclerosis. Cytokine Growth Factor Rev 2007;18:267-78.

91. Degraff DJ, Aguiar AA, Chen Q, Adams LK, Williams BJ, Sikes RA. Androgen mediated translational and postranslational regulation of IGFBP-2 in androgen-sensitive LNCaP human prostate cancer cells. Am J Transl Res 2010;2:200-8.

92. Martin JL, Baxter RC. Expression of insulin-like growth factor binding protein-2 by MCF-7 breast cancer cells is regulated through the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin pathway. Endocrinol 2007;148:2532-41.

93. Ernst CW, White ME. Hormonal regulation of IGF-binding protein-2 expression in proliferating C2C12 myoblasts. J Endocrinol 1996;149:417-29.

94. Hedbacker K, Birsoy K, Wysocki RW, Asilmaz E, Ahima RS, Farooqi IS, et al. Antidiabetic effects of IGFBP2, a leptin-regulated gene. Cell Metab 2010;11:11-22.

95. Wang GK, Hu L, Fuller GN, Zhang W. An interaction between insulin-like growth factor-binding protein 2 (IGFBP2) and integrin alpha5 is essential for IGFBP2-induced cell mobility. J Biol Chem 2006;281:14085-91.

96. Holmes KM, Annala M, Chua CY, Dunlap SM, Liu Y, Hugen N, et al. Insulin-like growth factor-binding protein 2-driven glioma progression is prevented by blocking a clinically significant integrin, integrin-linked kinase, and NF-kappaB network. Proc Natl Acad Sci U S A 2012;109:3475-80.

97. Fukushima T, Tezuka T, Shimomura T, Nakano S, Kataoka H. Silencing of insulin-like growth factor-binding protein-2 in human glioblastoma cells reduces both invasiveness and expression of progression-associated gene CD24. J Biol Chem 2007;282:18634-44.

98. Wolf E, Lahm H, Wu M, Wanke R, Hoeflich A. Effects of IGFBP-2 overexpression in vitro and in vivo. Pediatr Nephrol 2000;14:572-8.

99. Hoeflich A, Wu M, Mohan S, Foll J, Wanke R, Froehlich T, et al. Overexpression of insulin-like growth factor-binding protein-2 in transgenic mice reduces postnatal body weight gain. Endocrinol 1999;140:5488-96.

100. Wood TL, Rogler L, Streck RD, Cerro J, Green B, Grewal A, et al. Targeted disruption of IGFBP-2 gene. Growth Regul 1993;3:5-8.

101. Pintar JE, Schuller A, Cerro JA, Czick M, Grewal A, Green B. Genetic ablation of IGFBP-2 suggests functional redundancy in the IGFBP family. Progress in Growth Factor Research 1995;6:437-45.

102. Twigg SM, Baxter RC. Insulin-like growth factor (IGF)-binding protein 5 forms an alternative ternary complex with IGFs and the acid-labile subunit. J Biol Chem 1998;273:6074-9.

103. Fowlkes JL, Serra DM. Characterization of glycosaminoglycan-binding domains present in insulin-like growth factor-binding protein-3. J Biol Chem 1996;271:14676-9.

104. Schedlich LJ, Le Page SL, Firth SM, Briggs LJ, Jans DA, Baxter RC. Nuclear import of insulin-like growth factor binding protein-3 (IGFBP-3) and IGFBP-5 is mediated by the importin-b subunit. J Biol Chem 2000;275:23462-70.

105. Liu B, Lee HY, Weinzimer SA, Powell DR, Clifford JL, Kurie JM, et al. Direct functional interactions between insulin-like growth factor-binding protein-3 and retinoid X receptor-alpha regulate transcriptional signaling and apoptosis. J Biol Chem 2000;275:33607-13.

106. Leal SM, Liu Q, Huang SS, Huang JS. The type V transforming growth factor beta receptor is the putative insulin-like growth factor-binding protein 3 receptor. Journal of Biological Chemistry 1997;272:20572-6.

107. Huang SS, Ling TY, Tseng WF, Huang YH, Tang FM, Leal SM, et al. Cellular growth inhibition by IGFBP-3 and TGF-beta1 requires LRP-1. FASEB J 2003;17:2068-81.

108. Yamanaka Y, Fowlkes JL, Wilson EM, Rosenfeld RG, Oh Y. Characterization of insulin-like growth factor binding protein-3 (IGFBP-3) binding to human breast cancer cells: kinetics of IGFBP-3 binding and identification of receptor binding domain on the IGFBP-3 molecule. Endocrinol 1999;140:1319-28.

109. Ingermann AR, Yang YF, Han J, Mikami A, Garza AE, Mohanraj L, et al. Identification of a novel cell death receptor mediating IGFBP-3-induced anti-tumor effects in breast and prostate cancer. J Biol Chem 2010;285:30233-46.

110. Lee KW, Liu B, Ma L, Li H, Bang P, Koeffler HP, et al. Cellular internalization of insulin-like growth factor binding protein-3: distinct endocytic pathways facilitate re-uptake and nuclear localization. J Biol Chem 2004;279:469-76.

111. Wu HB, Kumar A, Tsai WC, Mascarenhas D, Healey J, Rechler MM. Characterization of the inhibition of DNA synthesis in proliferating mink lung epithelial cells by insulin-like growth factor binding protein-3. J Cell Biochem 2000;77:288-97.

112. Lemmey AB, Glassford J, Flick-Smith HC, Holly JMP, Pell JM. Differential regulation of tissue insulin-like growth factor binding protein (IGFBP)-3, IGF-I and IGF type 1 receptor mRNA levels, and serum IGF-I and IGFBP concentrations by growth hormone and IGF-I. J Endocrinol 1997;154:319-28.

113. Yamada PM, Lee KW. Perspectives in mammalian IGFBP-3 biology: local vs. systemic action. Am J Physiol Cell Physiol 2009;296:C954-C976.

114. Trump DL, Muindi J, Fakih M, Yu WD, Johnson CS. Vitamin D compounds: clinical development as cancer therapy and prevention agents. Anticancer Res 2006;26:2551-6.

115. Bushue N, Wan YJ. Retinoid pathway and cancer therapeutics. Adv Drug Deliv Rev 2010;62:1285-98.

116. Buckbinder L, Talbott R, Velasco-Miguel S, Takenaka I, Faha B, Seizinger BR, et al. Induction of the growth inhibitor IGF-binding protein 3 by p53. Nature 1995;377:646-9.

117. Duan C, Xu Q. Roles of insulin-like growth factor (IGF) binding proteins in regulating IGF actions. Gen Comp Endocrinol 2005;142:44-52.

118. Liu B, Lee KW, Li H, Ma L, Lin GL, Chandraratna RA, et al. Combination therapy of insulin-like growth factor binding protein-3 and retinoid X receptor ligands

synergize on prostate cancer cell apoptosis in vitro and in vivo. Clin Cancer Res 2005;11:4851-6.

119. Baxter RC. Signalling pathways involved in antiproliferative effects of IGFBP-3: a review. Molecular Pathology 2001;54:145-8.

120. Silha JV, Sheppard PC, Mishra S, Gui Y, Schwartz J, Dodd JG, et al. Insulin-like growth factor (IGF) binding protein-3 attenuates prostate tumor growth by IGF-dependent and IGF-independent mechanisms. Endocrinol 2006;147:2112-21.

121. Spagnoli A, Torello M, Nagalla SR, Horton WA, Pattee P, Hwa V, et al. Identification of STAT-1 as a molecular target of IGFBP-3 in the process of chondrogenesis. J Biol Chem 2002;277:18860-7.

122. Lee KW, Ma L, Yan X, Liu B, Zhang XK, Cohen P. Rapid apoptosis induction by IGFBP-3 involves an insulin-like growth factor-independent nucleomitochondrial translocation of RXRalpha/Nur77. J Biol Chem 2005;280:16942-8.

123. Paharkova-Vatchkova V, Lee KW. Nuclear export and mitochondrial and endoplasmic reticulum localization of IGF-binding protein 3 regulate its apoptotic properties. Endocr Relat Cancer 2010;17:293-302.

124. Butt AJ, Fraley KA, Firth SM, Baxter RC. IGF-binding protein-3-induced growth inhibition and apoptosis do not require cell surface binding and nuclear translocation in human breast cancer cells. Endocrinol 2002;143:2693-9.

125. Ricort JM, Binoux M. Insulin-like growth factor-binding protein-3 activates a phosphotyrosine phosphatase. Effects on the insulin-like growth factor signaling pathway. J Biol Chem 2002;277:19448-54.

126. Ning Y, Schuller AG, Bradshaw S, Rotwein P, Ludwig T, Frystyk J, et al. Diminished growth and enhanced glucose metabolism in triple knockout mice containing mutations of insulin-like growth factor binding protein-3, -4, and -5. Mol Endocrinol 2006;20:2173-86.

127. Murphy LJ, Molnar P, Lu X, Huang H. Expression of human insulin-like growth factor-binding protein-3 in transgenic mice. J Mol Endocrinol 1995;15:293-303.

128. Neuenschwander S, Schwartz A, Wood TL, Roberts J, Hennighausen L. Involution of the lactating mammary gland is inhibited by the IGF system in a trangenic mouse model. The Journal of Clinical Investigation 1996;97:2225-32.

129. Zhou R, Diehl D, Hoeflich A, Lahm H, Wolf E. IGF-binding protein-4: biochemical characteristics and functional consequences. J Endocrinol 2003;178:177-93.

130. Damon SE, Maddison L, Ware JL, Plymate SR. Overexpression of an inhibitory insulin-like growth factor binding protein (IGFBP), IGFBP-4, delays onset of prostate tumor formation. Endocrinol 1998;139:3456-64.

131. Ryan AJ, Napoletano S, Fitzpatrick PA, Currid CA, O'Sullivan NC, Harmey JH. Expression of a protease-resistant insulin-like growth factor-binding protein-4

inhibits tumour growth in a murine model of breast cancer. Br J Cancer 2009;101:278-86.

132. Zhou R, Flaswinkel H, Schneider MR, Lahm H, Hoeflich A, Wanke R, et al. Insulin-like growth factor-binding protein-4 inhibits growth of the thymus in transgenic mice. J Mol Endocrinol 2004;32:349-64.

133. Andress DL. Heparin modulates the binding of insulin-like growth factor (IGF) binding protein-5 to a membrane protein in osteoblastic cells. Journal of Biological Chemistry 1995;270:28289-96.

134. Amaar YG, Thompson GR, Linkhart TA, Chen ST, Baylink DJ, Mohan S. Insulin-like growth factor-binding protein 5 (IGFBP-5) interacts with a four and a half LIM protein 2 (FHL2). J Biol Chem 2002;277:12053-60.

135. Amaar YG, Baylink DJ, Mohan S. Ras-association domain family 1 protein, RASSF1C, is an IGFBP-5 binding partner and a potential regulator of osteoblast cell proliferation. J Bone Miner Res 2005;20:1430-9.

136. Schneider MR, Wolf E, Hoeflich A, Lahm H. IGF-binding protein-5: flexible player in the IGF system and effector on its own. J Endocrinol 2002;172:423-40.

137. Rho SB, Dong SM, Kang S, Seo SS, Yoo CW, Lee DO, et al. Insulin-like growth factor-binding protein-5 (IGFBP-5) acts as a tumor suppressor by inhibiting angiogenesis. Carcinogenesis 2008;29:2106-11.

138. Miyake H, Nelson C, Rennie PS, Gleave ME. Overexpression of insulin-like growth factor binding protein-5 helps accelerate progression to androgen-independence in the human prostate LNCaP tumor model through activation of phosphatidylinositol 3' -kinase pathway. Endocrinol 2000;141:2257-65.

139. Salih DA, Tripathi G, Holding C, Szestak TA, Gonzalez MI, Carter EJ, et al. Insulin-like growth factor-binding protein 5 (Igfbp5) compromises survival, growth, muscle development, and fertility in mice. Proc Natl Acad Sci U S A 2004;101:4314-9.

140. Bach LA. IGFBP-6 five years on; not so 'forgotten'? Growth Horm IGF Res 2005;15:185-92.

141. Grellier P, Berrebi D, Peuchmaur M, Babajko S. The IGF system in neuroblastoma xenografts: focus on IGF-binding protein-6. J Endocrinol 2002;172:467-76.

142. Kuo YS, Tang YB, Lu TY, Wu HC, Lin CT. IGFBP-6 plays a role as an oncosuppressor gene in NPC pathogenesis through regulating EGR-1 expression. J Pathol 2010;222:299-309.

143. Bienvenu G, Seurin D, Grellier P, Froment P, Baudrimont M, Monget P, et al. Insulin-like growth factor binding protein-6 transgenic mice: postnatal growth, brain development, and reproduction abnormalities. Endocrinol 2004;145:2412-20.

144. Pintar J, Schuller A, Bradshaw S, Cerro J, Grewal A. Genetic disruption of IGF binding proteins. In *Molecular mechanisms to regulate the activities of insulin-like growth factors*; Takano K, Hizuka N, Takahashi SI, Eds.; Elsevier: Amsterdam, 1998;1151,394 pages.

145. Hossenlopp P, Segovia B, Lassarre C, Roghani M, Bredon, Binoux M. Evidence of enzymatic degradation of insulin-like growth factor-binding proteins in the 150K complex during pregnancy. Journal of Clinical Endocrinology & Metabolism 1990;71:797-805.

146. Lawrence JB, Oxvig C, Overgaard MT, Sottrup-Jensen L, Gleich GJ, Hays LG, et al. The insulin-like growth factor (IGF)-dependent IGF binding protein-4 protease secreted by human fibroblasts is pregnancy-associated plasma protein-A. Proc Natl Acad Sci U S A 1999;96:3149-53.

147. Lalou C, Lassarre C, Binoux M. A proteolytic fragment of insulin-like growth factor (IGF) binding protein-3 that fails to bind IGFs inhibits the mitogenic effects of IGF-I and insulin. Endocrinol 1996;137:3206-12.

148. Baxter RC. Circulating levels and molecular distribution of the acid-labile (alpha) subunit of the high molecular weight insulin-like growth factor-binding protein complex. J Clin Endocrinol Metab 1990;70:1347-53.

149. Dai J, Scott CD, Baxter RC. Regulation of the acid-labile subunit of the insulin-like growth factor complex in cultured rat hepatocytes. Endocrinol 1994;135:1066-72.

150. Baxter RC, Martin JL, Beniac VA. High molecular weight insulin-like growth factor binding protein complex. Purification and properties of the acid-labile subunit from human serum. J Biol Chem 1989;264:11843-8.

151. Zapf J, Hauri C, Waldvogel M, Froesch ER. Acute metabolic effects and half-lives of intravenously administered insulinlike growth factors I and II in normal and hypophysectomized rats. J Clin Invest 1986;77:1768-75.

152. Ueki I, Ooi GT, Tremblay ML, Hurst KR, Bach LA, Boisclair YR. Inactivation of the acid labile subunit gene in mice results in mild retardation of postnatal growth despite profound disruptions in the circulating insulin-like growth factor system. Proc Natl Acad Sci U S A 2000;97:6868-73.

153. Payet LD, Firth SM, Baxter RC. The role of the acid-labile subunit in regulating insulin-like growth factor transport across human umbilical vein endothelial cell monolayers. J Clin Endocrinol Metab 2004;89:2382-9.

154. Hwa V, Oh Y, Rosenfeld RG. The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocr Rev 1999;20 :761-87.

155. Oh Y, Nagalla S, Yamanaka Y, Kim HS, Wilson E, Rosenfeld RG. Synthesis and characterization of insulin-like growth factor binding protein (IGFBP)-7. J Biol Chem 1996;271:30322-5.

156. Yamanaka Y, Wilson EM, Rosenfeld RG, Oh Y. Inhibition of insulin receptor activation by insulin-like growth factor binding proteins. Journal of Biological Chemistry 1997;272:30729-34.

157. Ma Y, Lu B, Ruan W, Wang H, Lin J, Hu H, et al. Tumor suppressor gene insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) induces senescence-like growth arrest in colorectal cancer cells. Exp Mol Pathol 2008;85:141-5.

158. Zuo S, Liu C, Wang J, Wang F, Xu W, Cui S, et al. IGFBP-rP1 induces p21 expression through a p53-independent pathway, leading to cellular senescence of MCF-7 breast cancer cells. J Cancer Res Clin Oncol 2012;138:1045-55.

159. Sell C, Rubini M, Rubin R, Liu JP, Efstratiadis A, Baserga R. Simian virus 40 large tumor antigen is unable to transform mouse embryonic fibroblasts lacking type 1 insulin-like growth factor receptor. Proc Natl Acad Sci U S A 1993;90:11217-21.

160. Florini JR, Ewton DZ, Coolican SA. Growth hormone and the insulin-like growth factor system in myogenesis. Endocr Rev 1996;17:481-517.

161. Sell C, Dumenil G, Deveaud C, Miura M, Coppola D, DeAngelis T, et al. Effect of a null mutation of the insulin-like growth factor I receptor gene on growth and transformation of mouse embryo fibroblasts. Mol Cell Biol 1994;14:3604-12.

162. Baserga R, Prisco M, Yuan T. IGF-1 receptor signaling in health and disease. 2002;120-40.

163. Carboni JM, Lee AV, Hadsell DL, Rowley BR, Lee FY, Bol DK, et al. Tumor development by transgenic expression of a constitutively active insulin-like growth factor I receptor. Cancer Res 2005;65:3781-7.

164. DiGiovanni J, Kiguchi K, Frijhoff A, Wilker E, Bol DK, Beltran L, et al. Deregulated expression of insulin-like growth factor 1 in prostate epithelium leads to neoplasia in transgenic mice. Proc Natl Acad Sci USA 2000;97:3455-60.

165. Resnicoff M, Sell C, Rubini M, Coppola D, Ambrose D, Baserga R, et al. Rat glioblastoma cells expressing an antisense RNA to the insulin-like growth factor-1 (IGF-1) receptor are nontumorigenic and induce regression of wild-type tumors. Cancer Res 1994;54:2218-22.

166. Yang XF, Beamer W, Huynh HT, Pollak M. Reduced growth of human breast cancer xenografts in hosts homozygous for the *'lit'* mutation. Cancer Res 1996;56:1509-11.

167. Dunn SE, Kari FW, French J, Leininger JR, Travlos G, Wilson R, et al. Dietary restriction reduces IGF-I levels, which modulates apoptosis, cell proliferation, and tumor progression in p53 deficient mice. Cancer Res 1997;57:4667-72.

168. Warren RS, Yuan H, Matli MR, Ferrara N, Donner DB. Induction of vascular endothelial growth factor by insulin-like growth factor 1 in colorectal carcinoma. J Biol Chem 1996;271:29483-8.

169. Goad DL, Rubin J, Wang H, Tashjian AH, Jr., Patterson C. Enhanced expression of vascular endothelial growth factor in human SaOS-2 osteoblast-like cells and murine osteoblasts induced by insulin-like growth factor I. Endocrinol 1996;137:2262-8.

170. Bermont L, Lamielle F, Fauconnet S, Esumi H, Weisz A, Adessi GL. Regulation of vascular endothelial growth factor expression by insulin-like growth factor-I in endometrial adenocarcinoma cells. Int J Cancer 2000;85:117-23.

171. Stoeltzing O, Liu W, Reinmuth N, Fan F, Parikh AA, Bucana CD, et al. Regulation of hypoxia-inducible factor-1alpha, vascular endothelial growth factor, and angiogenesis by an insulin-like growth factor-I receptor autocrine loop in human pancreatic cancer. Am J Pathol 2003;163:1001-11.

172. Tang X, Zhang Q, Shi S, Yen Y, Li X, Zhang Y, et al. Bisphosphonates suppress insulin-like growth factor 1-induced angiogenesis via the HIF-1alpha/VEGF signaling pathways in human breast cancer cells. Int J Cancer 2010;126:90-103.

173. Long L, Rubin R, Baserga R, Brodt P. Loss of the metastatic phenotype in murine carcinoma cells expressing an antisense RNA to the insulin-like growth factor receptor. Cancer Res 1995;55:1006-9.

174. Dunn SE, Ehrlich M, Sharp NJ, Reiss K, Solomon G, Hawkins R, et al. A dominant negative mutant of the insulin-like growth factor-I receptor inhibits the adhesion, invasion, and metastasis of breast cancer. Cancer Res 1998;58:3353-61.

175. Sachdev D, Hartell JS, Lee AV, Zhang X, Yee D. A dominant negative type I insulin-like growth factor receptor inhibits metastasis of human cancer cells. J Biol Chem 2004;279:5017-2024.

176. Hankinson SE, Willett WC, Colditz GA, Hunter DJ, Michaud DS, Deroo B, et al. Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. Lancet 1998;351:1393-6.

177. Giovannucci E, Pollak MN, Platz EA, Willett WC, Stampfer MJ, Majeed N, et al. A prospective study of plasma insulin-like growth factor-1 and binding protein-3 and risk of colorectal neoplasia in women. Cancer Epidemiol Biomarkers Prev 2000;9:345-9.

178. Chan JM, Stampfer MJ, Giovannucci E, Gann PH, Ma J, Wilkinson P, et al. Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. Science 1998;279:563-6.

179. Guevara-Aguirre J, Balasubramanian P, Guevara-Aguirre M, Wei M, Madia F, Cheng CW, et al. Growth hormone receptor deficiency is associated with a major

reduction in pro-aging signaling, cancer, and diabetes in humans. Sci Transl Med 2011;3:70ra13.

180. Hortobagyi GN. Trastuzumab in the treatment of breast cancer. N Engl J Med 2005;353:1734-6.

181. Gualberto A. Figitumumab (CP-751,871) for cancer therapy. Expert Opin Biol Ther 2010;10:575-85.

182. Olmos D, Postel-Vinay S, Molife LR, Okuno SH, Schuetze SM, Paccagnella ML, et al. Safety, pharmacokinetics, and preliminary activity of the anti-IGF-1R antibody figitumumab (CP-751,871) in patients with sarcoma and Ewing's sarcoma: a phase 1 expansion cohort study. Lancet Oncol 2010;11:129-35.

183. Jassem J, Langer CJ, Karp DD, Mok T, Benner RJ, Green SJ, et al. Randomized, open label, phase III trial of figitumumab in combination with paclitaxel and carboplatin versus paclitaxel and carboplatin in patients with non-small cell lung cancer (NSCLC). J Clin Oncol 2010;28:15s (suppl; abstr 7500).

184. Yin D, Sleight B, Alvey C, Hansson AG, Bello A. Pharmacokinetics and Pharmacodynamics of Figitumumab, a Monoclonal Antibody Targeting the Insulin-Like Growth Factor 1 Receptor, in Healthy Participants. J Clin Pharmacol 2012; published online DOI: 10.1177/0091270011432934.

185. Moreau P, Cavallo F, Leleu X, Hulin C, Amiot M, Descamps G, et al. Phase I study of the anti insulin-like growth factor 1 receptor (IGF-1R) monoclonal antibody, AVE1642, as single agent and in combination with bortezomib in patients with relapsed multiple myeloma. Leukemia 2011;25:872-4.

186. Pappo AS, Patel SR, Crowley J, Reinke DK, Kuenkele KP, Chawla SP, et al. R1507, a monoclonal antibody to the insulin-like growth factor 1 receptor, in patients with recurrent or refractory Ewing sarcoma family of tumors: results of a phase II Sarcoma Alliance for Research through Collaboration study. J Clin Oncol 2011;29:4541-7.

187. Ramalingam SS, Spigel DR, Chen D, Steins MB, Engelman JA, Schneider CP, et al. Randomized phase II study of erlotinib in combination with placebo or R1507, a monoclonal antibody to insulin-like growth factor-1 receptor, for advanced-stage non-small-cell lung cancer. J Clin Oncol 2011;29:4574-80.

188. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. Nat Rev Cancer 2008;8:915-28.

189. Wittman M, Carboni J, Attar R, Balasubramanian B, Balimane P, Brassil P, et al. Discovery of a (1H-benzoimidazol-2-yl)-1H-pyridin-2-one (BMS-536924) inhibitor of insulin-like growth factor I receptor kinase with in vivo antitumor activity. J Med Chem 2005;48:5639-43.

190. Macaulay VM, Middleton MR, Eckhardt SG, Juergens RA, Stephens AW, Poondru S, et al. Phase I study of OSI-906, dual tyrosine kinase inhibitor of insulin-like growth factor-1 receptor (IGF-1R) and insulin receptor (IR) in

combination with erlotinib (E) in patients with advanced solid tumors. J Clin Oncol 2010;28:15s-(suppl; abstr 3016).

191. Meyer GE, Chesler L, Liu D, Gable K, Maddux BA, Goldenberg DD, et al. Nordihydroguaiaretic acid inhibits insulin-like growth factor signaling, growth, and survival in human neuroblastoma cells. J Cell Biochem 2007;102:1529-41.

192. Pollak M. The insulin receptor/insulin-like growth factor receptor family as a therapeutic target in oncology. Clin Cancer Res 2012;18:40-50.

193. Dool C, Mashhedi H, Zakikhani M, David S, Zhao Y, Birman E, et al. IGF-1/insulin receptor kinase inhibition by BMS-536924 is better tolerated than alloxan-induced hypoinsulinemia and more effective than metformin in the treatment of experimental insulin responsive breast cancer. Endocr Relat Cancer 2011;18:699-709.

194. Shojaei F. Anti-angiogenesis therapy in cancer: current challenges and future perspectives. Cancer Lett 2012;320:130-7.

195. Pietrzkowski Z, Wernicke D, Porcu P, Jameson BA, Baserga R. Inhibition of cellular proliferation by peptide analogues of insulin-like growth factor 1. Cancer Res 1992;52:6447-51.

196. Gao J, Chesebrough JW, Cartlidge SA, Ricketts SA, Incognito L, Veldman-Jones M, et al. Dual IGF-I/II-neutralizing antibody MEDI-573 potently inhibits IGF signaling and tumor growth. Cancer Res 2011;71:1029-40.

197. Adam PJ, Ostermann E, Lamche HR, Hofmann MH, Kroez M, Borges E, et al. Pharmacodynamic properties and antitumor efficacy of BI 836845, a fully human IGF ligand neutralizing antibody. Proccedings of AACR-NCI-EORTC International Conference: Molecular Targets and Cancer Therapeutics 2011;2011 Nov 12-16; San Francisco, CA.:Philadelphia (PA): AACR; Mol Cancer Ther 2011; 10(11 Suppl)-Abstr nr A208.

Chapter 2

IGFBP-2 expression in MCF-7 cells is regulated by the PI3K/AKT/mTOR pathway through Sp1 induced increase in transcription.

This section is based on the following published article:

Mireuta M, Darnel A, Pollak M. IGFBP-2 expression in MCF-7 cells is regulated by the PI3K/AKT/mTOR pathway through Sp1-induced increase in transcription. *Growth Factors* 28: 243-55, 2010.

Preface

For the first part of the experimental work of the thesis, we examine aspects of the regulation of IGFBP-2 in neoplasia. As discussed in the literature review, IGFBPs have complex roles in addition to their classical actions as carrier proteins for IGFs. Additionally, there is epidemiologic evidence suggesting increased circulating IGFBP-2 in patients with various cancers. Increased expression of IGFBP-2 by tumors has also been shown consistently in many malignancies, including breast. We had evidence, both from literature and previous work in our laboratory, that the PI3K/AKT/mTOR pathway is an important regulator of IGFBP-2 expression at the protein level. The fact that this pathway is frequently overactive in neoplasia may explain part of the epidemiologic results.

However, there were several gaps in knowledge with respect to the underlying mechanisms responsible for increased IGFBP-2 protein levels. We investigated the effect of the PI3K/AKT/mTOR pathway activation on IGFBP-2 mRNA levels and gene transcription in a breast cancer cell line.

2.1 Abstract

IGF binding protein 2 (IGFBP-2) has been implicated in the pathophysiology of neoplasia. The PI3K/AKT/mTOR pathway has recently been shown to be a predominant regulator of IGFBP-2 at the protein level in MCF-7 breast cancer cells. However, there are gaps in knowledge with respect to the molecular mechanisms that underlie this regulation. Here we show that the PI3K/AKT/mTOR pathway regulates IGFBP-2 protein levels by modulating IGFBP-2 mRNA abundance in MCF-7 cells. This change is achieved by regulating transcription through a critical region present in the first 200 bp upstream of the transcription initiation site where Sp1 transcription factor binds and drives transcription. IGF-1 treatment leads to increased nuclear abundance of Sp1 and increased IGFBP-2 mRNA and protein levels. Rapamycin and LY294002 induce a decline in Sp1 nuclear abundance and IGFBP-2 mRNA and protein levels. This work provides a mechanistic explanation for the observed effects of the PI3K/AKT/mTOR pathway on IGFBP-2 levels in MCF-7 cells.

2.2 Introduction

Insulin-like growth factor binding protein 2 (IGFBP-2) is a member of the family of insulin-like growth factor binding proteins, of which IGFBP-1 through 6 have been relatively well characterized. Although they are expressed in many tissues, IGFBPs have classically been defined as carrier proteins for insulin-like growth factors (IGFs) in the blood. They are approximately 36 kDa proteins and share common structural motifs, with conserved N and C-termini involved in IGF binding and a variable central L (linker)-domain containing several posttranslational modification sites. It is thought that IGFBPs function in the circulation to regulate IGF bioavailability and half-life. IGFBP-3 is the most abundant IGFBP in the blood, binding IGFs as a trimer with ALS (acid-labile subunit). Other IGFBPs are present at lower concentrations (1-5). However, IGFBP-2 is the most abundant IGFBP in other fluids such as CSF and seminal plasma (6).

Despite their relatively well characterized functions in the blood, IGFBPs have more obscure functions in the local microenvironment of cells. They can inhibit actions of IGF-1 and IGF-2 by binding and sequestering them away from the IGF-1 receptor. At the same time, they can potentiate IGF actions by possibly acting as chaperones and binding cell surface structures and bringing IGFs in close proximity to the IGF-1R. Furthermore, they can act independently of IGFs by putative mechanisms such as direct integrin binding (RGD domains) or possibly specific receptors (7-12). Further complexity arises from the fact that IGFBPs L-domain exhibits protease cleavage, glycosylation and phosphorylation sites which serve to regulate their functions even further (13-15). Moreover, multiple sized fragments released from IGFBP proteolysis have been reported to have biological significance (16).

Depending on the experimental model used, IGFBP-2 has been reported, like other IGFBPs, to potentiate, inhibit or act independently of IGFs in vitro (17-22).

Epidemiologic studies have shown IGFBP-2 to be upregulated at the protein level in many malignancies and to be positively correlated with tumor progression in many cancers, including breast (23-28). It is, however, unclear if IGFBP-2 is merely a marker of malignant cell behavior or if it contributes to tumor growth. As an initial step towards understanding the actions of IGFBP-2, in the context of its overexpression in neoplasia, it is important to understand pathways responsible for its regulation.

Several publications have examined the regulation of IGFBP-2 in different cell types and have reported modulation by factors including IGF-1, insulin and steroids (29-32). The PI3K/AKT/mTOR pathway is one of the most upregulated pathways in neoplastic cells through mechanisms such as PTEN loss of function or PI3K activating mutations (33). This pathway may be predominant in the regulation of IGFBP-2 at the protein level. Studies have shown that activation of the PI3K/AKT/mTOR pathway leads to overexpression of IGFBP-2 in experimental models, including the MCF-7 breast cancer cell line (34,35). However, there are still major gaps in knowledge with respect to the molecular mechanisms employed to regulate IGFBP-2 protein levels. Our goal was to extend current knowledge of the IGFBP-2 regulation by the PI3K/AKT/mTOR pathway and to propose a mechanistic explanation for the observed effects at the protein level.

2.3 Materials and Methods

Cell lines and Materials

The MCF-7 and T47D cell lines were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured in standard RPMI medium supplemented with 10% fetal bovine serum and 20 ug/ml Gentamycin. IGF-1, EGF, and insulin were purchased from Cell Sciences (Canton, MA, USA), LY294002, rapamycin, PD98059 and SB203580 were purchased from Calbiochem (San Diego, CA, USA). Mithramycin A was purchased from BIOMOL (Zandhoven, Belgium).

ELISA

Conditioned medium from MCF-7 cells was collected, diluted 30 times in RPMI Serum Free Medium and IGFBP-2 concentration was measured by ELISA. The ELISA components (antibodies, standards etc) were purchased from RnD Systems (Mineapolis, MN, USA) while ELISA 96-well plates were purchased from Costar (Lowell, MA, USA). Manipulations were performed according to manufacturer's instructions. IGFBP-2 concentrations in conditioned media are not corrected for cell number, as the steady states reached under each treatment were independent of cell number. We believe that these steady states are reached as a consequence of the system achieving an equilibrium between secretion and degradation rates. The measurements are thus left as concentration per well.

RNA collection and qRT-PCR

RNA was collected from MCF-7 cells using TRizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. Then, 5ug of total RNA was reverse transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase and random primers (Invitrogen, Carlsbad, CA, USA). To eliminate the RNA template, RNase H was added to the reaction in the final step. Then, total cDNA was purified from the reaction mixture using silica columns (Qiagen,

Venlo, The Netherlands). Concentrations of the relatively pure cDNA were assessed using absorbance spectrometry (Nanodrop) and 500ng of total cDNA were used downstream in the qRT-PCR experiments. Taqman probe, enzyme mix and 96-well plates were purchased from Applied Biosystems (Foster City, CA, USA) and qRT-PCR was performed on a Light Cycler. Results were compared to a standard curve of known amounts of IGFBP-2 DNA and analyzed with manufacturer's software.

Luciferase constructs and Sp1 plasmids

Respectively, a 2.4 Kbp and 1.2 Kbp region upstream of the IGFBP-2 translation start site was cloned into the PGL-3 luciferase enhancer vector (Promega, Madison, WI, USA). The 2.4 Kbp region was cloned by PCR using forward primer :5'- CGTTTGCGATTTGCAGTAGA-3' and reverse primer : 5'-CTCCTCCGCTTCTTCCTCCT-3' while the 1.2Kbp sequence was cloned using forward primer: 5'- GTGGAGGCAGCTTAATGGTC-3' and reverse primer 5'-CTCCTCCGCTTCTTCCTCCT-3'. Bgl-2 and Kpn-1 sites were introduced into the primers and the amplification product was cloned utilizing the same restriction sites in the vector. The 700bp and 200bp sequences were generated using restriction enzymes Xho-1 and Sma-1 in the 1.2 Kbp construct and ligated at the same sites into the vector.

pPacSp1 vector contains a sequence coding for a truncated version of the transcription factor Sp1, which has been shown to maintain at least 90% of full-length Sp1 function(36). pPac0 is an empty vector and served as control. Both plasmids were purchased from Addgene (Cambridge, MA, USA).

Cell lysates and nuclear/cytoplasmic fractionation

Total cell lysates were obtained using RIPA buffer as described previously (34). Nuclear lysates were obtained by using three buffers: Buffer A (50 mM NaCl, 10 mM HEPES, 500mM sucrose, 1mM EDTA, 0.5 mM spermidine, 0.15mM spermine, 0.2 % TX-100, 7mM mercaptoethanol), Buffer B (50 mM NaCl, 10 mM HEPES, 25% glycerol, 1mM EDTA, 0.5 mM spermidine, 0.15mM spermine, 7mM

mercaptoethanol), Buffer C (350 mM NaCl, 10 mM HEPES, 25% glycerol, 1mM EDTA, 0.5 mM spermidine, 0.15mM spermine, 7mM mercaptoethanol) all supplemented with protease inhibitors. Briefly, cells were collected and lysed with Buffer A for 2 minutes and then centrifuged. The supernatant was termed cytoplasmic extract. Subsequently, the precipitate was washed twice in Buffer B for 2 minutes and was then lysed using Buffer C on ice for 30 min. Final centrifugation yielded nuclear extracts.

Transfections, Western Blots and Luciferase measurements

Transfections were done using electroporation (Microporator, Digital Bio Technology, Seoul, Korea) and cells were cultured in serum containing RPMI medium for 24 hours. Cells were then incubated in serum free medium containing treatments and left in culture for 72 hours until measurements were performed.

Luciferase measurements were done on a luminometer following addition of Bright-GLO luciferase substrate solution (Promega, Madison, WI, USA) to the media according to manufacturer's specifications.

For Western Blots, total protein from lysates was quantified (BioRad, Hercules, CA, USA). 30ug of total protein was loaded per lane and was further transferred onto a nitrocellulose membrane. These were immunoblotted with specific antibodies for Sp1, α-tubulin, lamin A/C (Santa Cruz, Santa Cruz, CA, USA), phospho-serine 473 AKT, AKT, p70 S6 Kinase, phospho-threonine 389 p70 S6 Kinase and beta-actin (Cell Signaling, Danvers, MA, USA). Finally, membranes were exposed on chemiluminescence film.

Immunofluorescence

Cells were washed three times in PBS for 5 minutes each time, fixed with paraformaldehyde for 10 minutes and washed again three times in PBS. Then, cells were incubated for 30 minutes in PBS containing 0.5% BSA and 0.5% Triton-X. Anti-Sp1 antibody was diluted 1:100 in PBS containing 0.5% BSA and 0.5% Triton-X and applied overnight in a humidified chamber. The following day,

cells were washed three times in PBS and the fluorochrome conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) was added at a 1:1000 dilution in PBS containing BSA and Triton-X for one hour. Cells were then washed three times in PBS and mounted with media containing DAPI (Vectashield, Burlingame, CA, USA). The slides were visualized using a Leica microscope and appropriate software.

Immunoprecipitation and Chromatin Immunoprecipitation

For immunoprecipitation of Sp1, cells were lysed using RIPA buffer supplemented with protease and phosphatase inhibitors (Roche, Basel, Switzerland). Then, 500 ug of total cellular protein was incubated for 1 hour with 40 ul protein A/G conjugated beads (Santa Cruz, Santa Cruz, CA, USA) for preclearance at 4^{o}C. Beads were removed by centrifugation and the lysates were further incubated with 1ug Sp1 specific antibody (Santa Cruz, Santa Cruz, CA, USA) for 1 hour at 4^{o}C. Afterwards, 50 ul of protein A/G conjugated agarose beads were added to each tube and incubated overnight on a rotating device at 4^{o}C. The following day, the tubes were washed once in 1 ml of RIPA buffer supplemented with protease and phosphatase inhibitors (centrifugation at 9000 rpm for 1 minute) and final resuspension was done in SDS-PAGE 6X concentrated loading buffer. Samples were boiled for 5-10 minutes and loaded on a SDS-PAGE gel. After transfer, nitrocellulose membranes were immunoblotted with specific antibodies for SUMO, phospho-threonine, phospho-serine, phospho-tyrosine (Santa Cruz, Santa Cruz, CA, USA) and O-linked N Acetylglucosamine (Pierce, Rockford, IL, USA).

For chromatin immunoprecipitation, cells were fixed in 1% formaldehyde for 25 minutes, washed three times in PBS and lysed with RIPA buffer supplemented with protease and phosphatase inhibitors. Then, cells were sonicated on ice 12 times for 10 seconds each at 40% amplitude in a Sonic Dismembrator Model 500 (Fisher Scientific, Pittsburgh, PA, USA). Further, lysates were centrifuged at 13000 rpm 4^{o}C, and total protein levels in the supernatant were quantified. Three mg of total protein were incubated with 40 ul of protein A/G agarose (Santa Cruz,

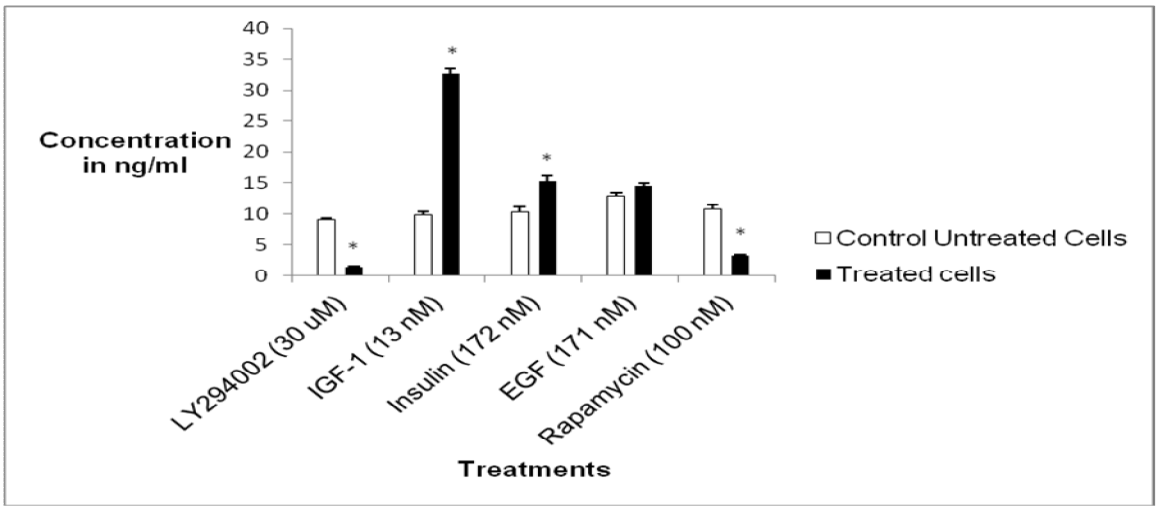
Santa Cruz, CA, USA) beads for preclearance for 2 hours at 4^{o}C in a rotating device. Beads were removed by centrifugation and the lysates were further incubated with 3ug anti-Sp1 antibody for 1h followed by addition of 70ul of protein A/G agarose beads and incubation overnight at 4^{o}C in a rotating device. The following day, beads were washed in RIPA three times and resuspended in a buffer containing 50 mM TRIS pH 8, 1mM EDTA, 1% SDS, 50 mM NaHCO3, 200 mM NaCl and 5mM DTT and incubated at 65^{o}C overnight with shaking. The following day, 2ul of a 10mg/ml Proteinase K stock solution were added and the tubes were further incubated at 42^{o}C for 2 hours. Finally, the DNA was extracted with phenol/chloroform/isoamyl alcohol solution twice and used as the input template in a conventional PCR reaction for 35 cycles. The primers used (left primer: 5' AGCAGGGAACCCCCAGAG -3' and right primer: 5' CTAAAGGGCCGGCTTCTC -3') are complementary to a region within 200 base pairs upstream of the IGFBP-2 transcription initiation site and produce a PCR product of 156bp.

2.4 Results

IGFBP-2 protein and mRNA are regulated through the PI3K/AKT/mTOR pathway in MCF-7 breast cancer cells

As IGFBP-2 protein levels have been previously shown to be modulated by the PI3K/AKT/mTOR pathway in MCF-7 cells, we wished to investigate if these effects also occur at the mRNA level. We treated MCF-7 cells with activators IGF-1, insulin, EGF and inhibitors LY294002 (PI3K inhibitor) and rapamycin (mTOR inhibitor) at concentrations previously reported to affect IGFBP-2 protein levels. We confirm that IGF-1 (13 nM) induces an approximate 3 fold increase in IGFBP-2 protein levels in conditioned media of MCF-7 cells and LY294002 treatment (30 uM) results in a decrease of about 7- fold while rapamycin (100 nM) induces a 2 fold decrease. Insulin (172 nM) and EGF (171 nM), despite hundred fold higher concentrations, provoke more modest changes in IGFBP-2 protein levels (about 50% increase by insulin and no statistically significant increase by EGF) (Figure 2.1A). IGFBP-2 protein levels as well as mRNA levels were measured at three time points : 24, 48 and 72 hours under all treatments. LY294002 and rapamycin treatment induced new steady states in both mRNA and protein levels after 24 hours of treatment which did not change at the subsequent 48 and 72 hours time points (data not shown). Similary, IGF-1 treatment induced new steady states of IGFBP-2 mRNA and protein levels after 48 hours of treatment (data not shown). Since our goal was to measure steady-state changes in IGFBP-2 mRNA and protein levels rather than short term effects, all subsequent measurements were performed after 72 hours of treatment, a time sufficient for cells to reach steady state levels of expression under all treatments.

A)



B)

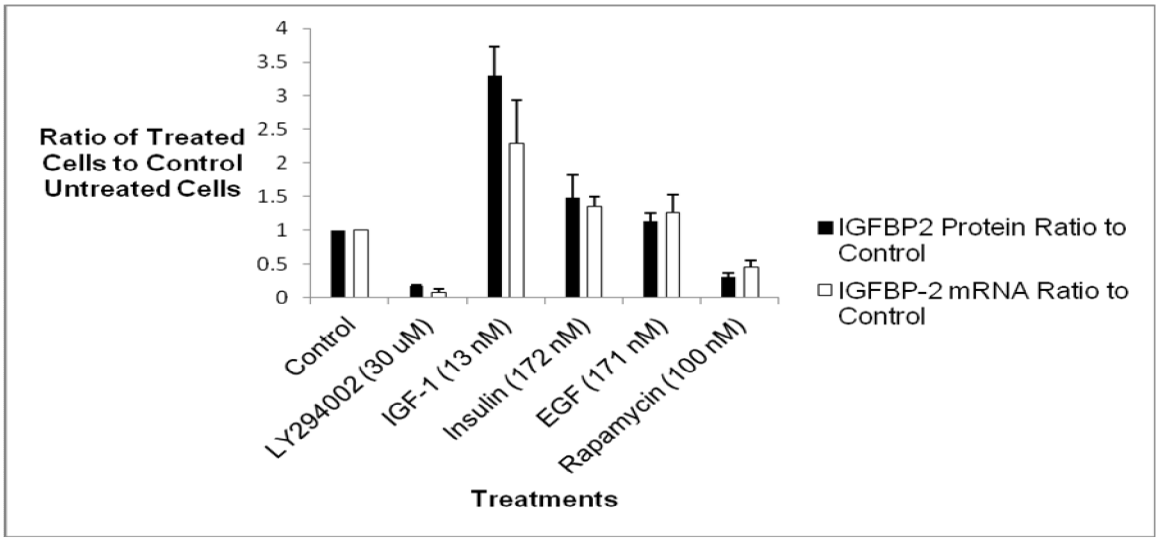


Figure 2.1: Effect of PI3K/AKT/mTOR pathway modulators on IGBP-2 protein and mRNA expression in MCF-7 breast cancer cells. A) Concentration of IGFBP-2 protein in conditioned media of MCF-7 cells. Cells were plated at $5x10^5$ cells per well in 6 well plates and cultured for 24 hours. The media was then exchanged for serum free media containing the desired concentrations of treatments (IGF-1 at 13 nm, LY294002 at 30uM, rapamycin at 100nM, insulin at 172 nM and EGF at 171 nM). Cells were further incubated for 72 hours following which media was collected and IGFBP-2 concentration was measured by ELISA. Concentration is expressed as ng/ml per well. * indicates statistical significance compared to control as obtained by ANOVA (n= 12 (in duplicate), p-value <0.0001) B) Ratios of IGFBP-2 mRNA and protein measurements compared to control untreated cells. Cells were cultured as above and IGFBP-2 mRNA was measured using qRT-PCR and is expressed as ratios of treated cells to control untreated cells. Protein measurements are the same as in A), but expressed as ratios of treated cells to control untreated cells. Error bars indicate approximate 95 % confidence intervals obtained by the delta method approximation to Fieller's method with n=6 for IGFBP-2 mRNA.

As shown in Figure 2.1B, IGFBP-2 mRNA, as measured by quantitative real-time PCR (qRT-PCR), varies in the same direction and with similar magnitude as the relative protein changes induced by the above-mentioned treatments. IGF-1 treatment results in the highest induction of IGFBP-2 mRNA (~2-fold) while LY294002 and rapamycin decrease IGFBP-2 mRNA levels by roughly 8-fold and 2-fold respectively. Insulin, as was the case for protein levels, induced a relatively small increase in mRNA levels (~25%), while EGF induced no statistically significant change.

Modulators of the PI3K/AKT/mTOR pathway act through DNA elements proximal to the transcription initiation site

In order to understand the effects of manipulation of the PI3K/AKT/mTOR pathway on IGFBP-2 mRNA regulation, we conducted a luciferase reporter experiment in order to isolate a region upstream of the IGFBP-2 transcription start site that might mediate the changes observed. We cloned several sequences proximal to the IGFBP-2 transcription start site, ranging from 2.4 Kbp to 200bp, using PCR on MCF-7 genomic DNA (Figure 2.2) directly upstream of a luciferase gene in the PGL-3 vector. MCF-7 cells transfected with the latter constructs were either treated or not with each modulator of the PI3K/AKT/mTOR pathway and three days later, the cells were lysed and their respective luciferase expression was measured. The changes observed are expressed as ratios of treated transfected cells to untreated transfected cells for each construct for both the activators and inhibitors of the pathway (Figure 2.3A, 2.3B). The change with each treatment correlates both in direction and magnitude with the change observed for IGFBP-2 mRNA levels, suggesting that these compounds affect mainly transcription of the IGFBP-2 gene rather than any other mechanism of mRNA regulation. As for IGFBP-2 mRNA levels, IGF-1 (13 nM) increases luciferase expression by roughly 2-fold whereas rapamycin (100 nM) and LY294002 (30 uM) decrease expression (1.5-fold and 5-fold respectively). Insulin (172 nM) and EGF (171 nM) do not induce any change in luciferase expression. Also, none of the treatments have an effect on the Promoterless construct nor on

the 1.2 Kbp Control construct, indicating that the effects observed are not due to nonspecific factors such as increased cell death or luciferase translational modulation. In absolute values, the Promoterless and 1.2 Control constructs displayed around 10% luminescence activity compared to the remaining three constructs and GFP transfected or untransfected cells displayed no luminescence activity (data not shown). Additionally, since the same change is present across all constructs for all treatments, the data suggest that the critical region present in the first 200 bp upstream of the transcription initiation site is responsible for the effects observed on the IGFBP-2 mRNA.

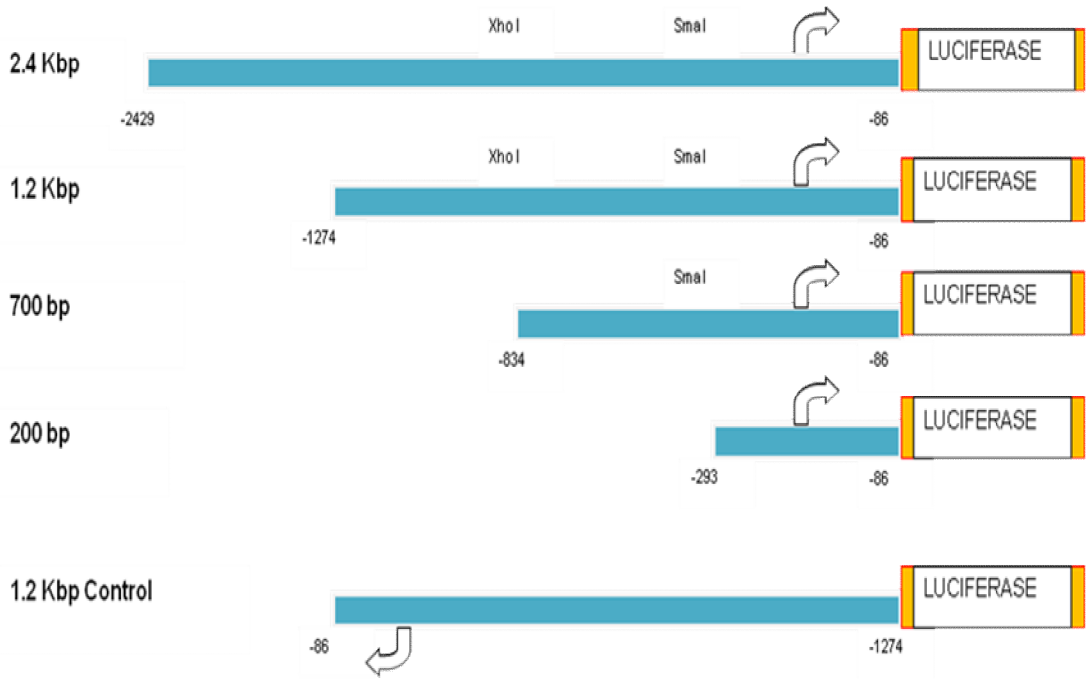
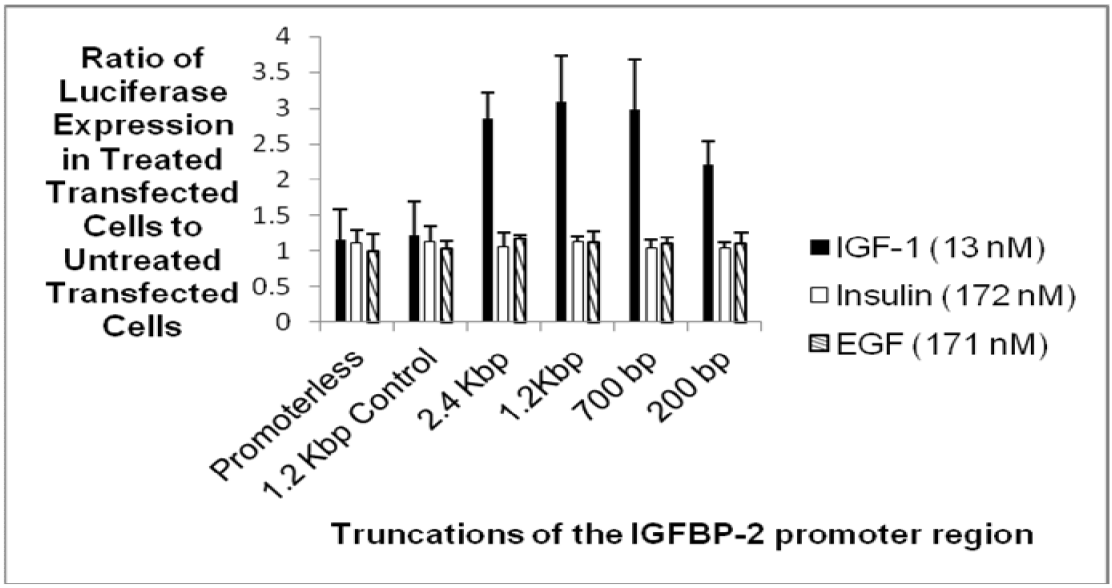


Figure 2.2: Schematic diagram of luciferase reporter assay constructs. A 2.4 and 1.2 Kbp sequence upstream of the translation initiation site were cloned into the PGL-3 vector. Positions relative to the first ATG of IGFBP-2 are indicated below, the arrow represents the IGFBP-2 transcription initiation site. The 1.2 Kbp Control construct was used as a negative control and represents the same sequence as the 1.2 Kbp construct but cloned in the reverse orientation.

A)



B)

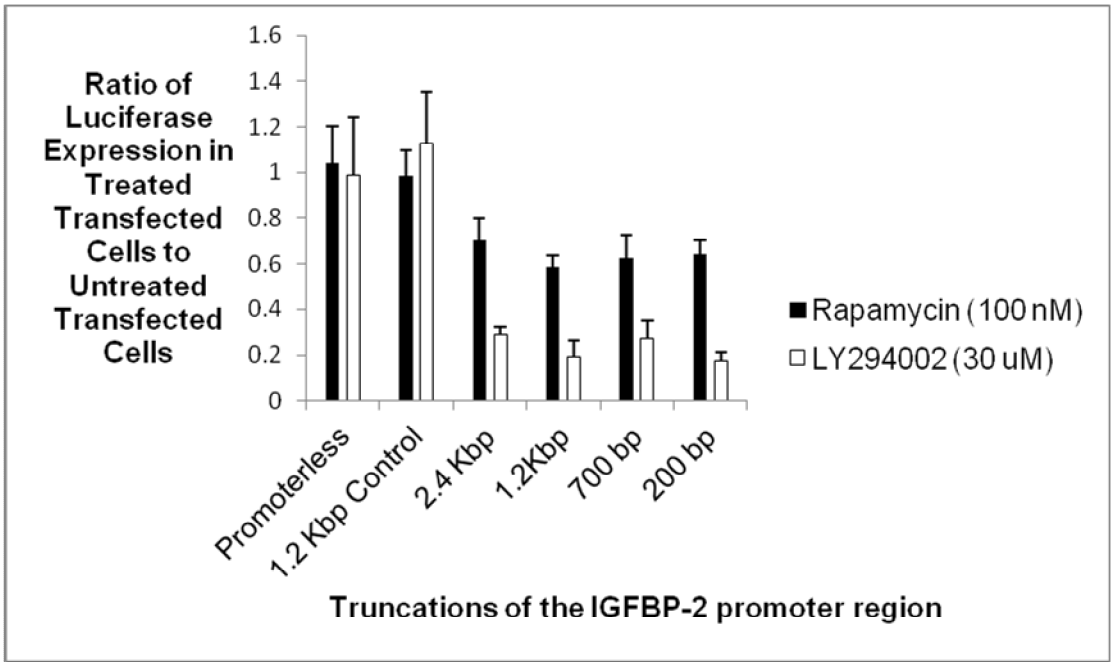


Figure 2.3: Effect of PI3K/AKT/mTOR pathway modulators on serially truncated IGFBP-2 promoter region sequences. A) Effect of activators of the pathway. $4x10^5$ MCF-7 cells were transfected with 2ug of DNA per well in 24-well plates using electroporation. Cells were cultured for 24 hours and then transferred to serum free conditions with and without the appropriate growth factors. Luciferase readings were performed 72 hours (steady-state) later. Ratios shown are luciferase readings of treated transfected cells to untreated transfected cells for each construct. Error bars represent approximate 95 % confidence intervals obtained by the delta method approximation to Fieller's method with n=6 (in duplicate). B) Effect of inhibitors of the pathway. Cells were treated exactly as in A.

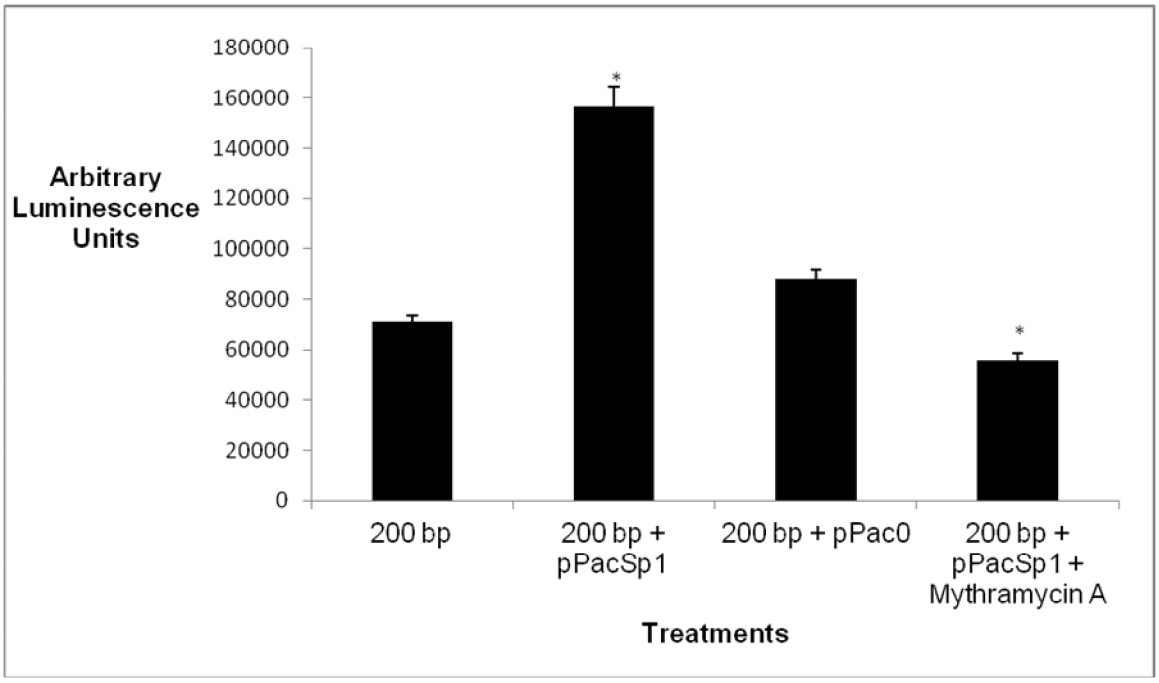
IGF-1, LY294002 and rapamycin modulate IGFBP-2 mRNA levels through Sp1

It has been previously shown for the rat IGFBP-2 gene that Sp1 or Sp1-like elements bind to four GC rich boxes situated in the proximal promoter region to drive transcription, although the study did not consider effects of any regulators of expression (37). Since these four boxes are almost identical in humans, and present in our 200bp construct, we hypothesized that Sp1 transcription factor could be modulated by the PI3K/AKT/mTOR pathway in a way that could explain our observations.

We overexpressed Sp1 in MCF-7 cells along with the 200bp sequence construct. Cotransfection of the 200bp construct with an Sp1 plasmid results in an increase in luciferase expression whereas cotransfection with a control plasmid does not induce any change. Also, the increase produced by Sp1 is abrogated when cells are treated with Mithramycin A (500nM), a compound which binds GC rich regions and inhibits Sp1 driven transcription (38) (Figure 2.4 A).

Furthermore, we performed a chromatin immunoprecipitation experiment in order to investigate the levels of Sp1 transcription factor bound to the region comprising the 4 GC rich boxes upstream of the IGFBP-2 transcription initiation site. We performed, in MCF-7 cells, an immunoprecipitation of total Sp1 cross-linked DNA followed by PCR amplification of the sequence containing the 4 GC rich boxes. Compared to samples for control untreated cells, samples from IGF-1 (13nM) treated cells displayed higher levels of PCR product, while samples from LY294002 (30 uM) and rapamycin (100 nM) treated cells displayed smaller levels of PCR product. Samples from insulin (172 nM) and EGF (171 nM) treated cells led to no apparent change in the levels of PCR product as compared to control (Figure 2.4B).

A)



B)

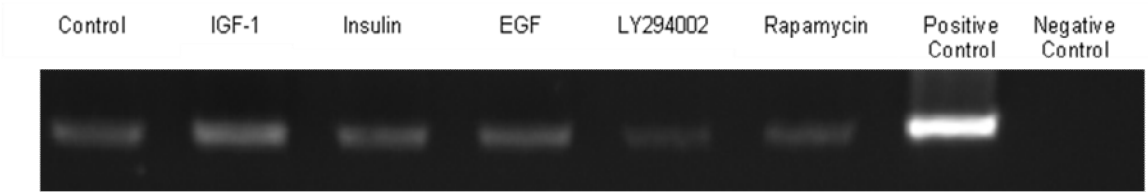


Figure 2.4: Effects of Sp1 on IGFBP-2 promoter transactivation. A) The effect of overexpression of Sp1 on the 200bp luciferase reporter construct. $4x10^5$ cells were transfected with 2ug of 200bp construct or cotransfected with the former and 2ug of pPac Sp1 or pPac 0 vector (Addgene) accordingly. Cells were left to incubate for 24 hours and transferred to serum free media with or without Mithramycin A (an Sp1 inhibitor) at 500nM for an additional 24 hours before luciferase measurements were performed. Error bars are the SEM and * represents statistical significance compared to control as obtained by ANOVA (n=6 (in duplicate), p-value < 0.001). B) Chromatin immunoprecipitation. Cells were cultured in 10% FBS media in15mm round dishes until 80% confluence was achieved, then treated in serum-free media for 72 hours with the different compounds. A chromatin immunoprecipitation experiment was carried out and the product was amplified by PCR. The lane termed positive control represents a PCR reaction with the 700bp construct as a template and the lane termed negative control represents a PCR reaction on a sample from control untreated cells where without anti-Sp1 primary antibody. The gel shown is representative of three independent experiments.

Nevertheless, none of the previous treatments affected Sp1 protein abundance in total cell extracts (Figure 2.5A). The normal functioning of Sp1, like that of many other transcription factors, requires shuttling of the transcription factor between the cytoplasm and nucleus. We therefore fractionated cells into nuclear and cytoplasmic extracts followed by immunoblotting (Figure 2.5B). Our results indicated that IGF-1 (13 nM) increases the nuclear proportion of Sp1, whereas LY294002 (30 uM) and rapamycin (100 nM) decrease it. Densitometry measurements indicated that the changes in nuclear Sp1 are correlated with those of luciferase expression and IGFBP-2 mRNA. IGF-1 increased Sp1 nuclear abundance by around 80% while rapamycin and LY294002 decreased it by 22% and 80 % respectively. Insulin (172 nM) and EGF (171 nM) treatments do not result in significant increase in nuclear Sp1 (Figure 2.5C, 2.5D).

Furthermore, we conducted an immunofluorescence experiment with MCF-7 cells in order to visualize the large changes in nuclear Sp1 abundance after exposure to IGF-1 or LY294002 and observed that nuclear Sp1 increases with IGF-1 exposure and decreases with LY294002 exposure, consistent with Western Blot results (Figure 2.6A). The magnitude of changes seen with rapamycin exposure by Western Blot was too small to be detected by immunofluorescence and no effect on nuclear Sp1 by insulin nor EGF was observed (data not shown). Similar results were observed with the breast cancer cell line T47D which was previously shown to regulate IGFBP-2 protein levels through the PI3K/AKT/mTOR pathway (35) (Figure 2.6B).

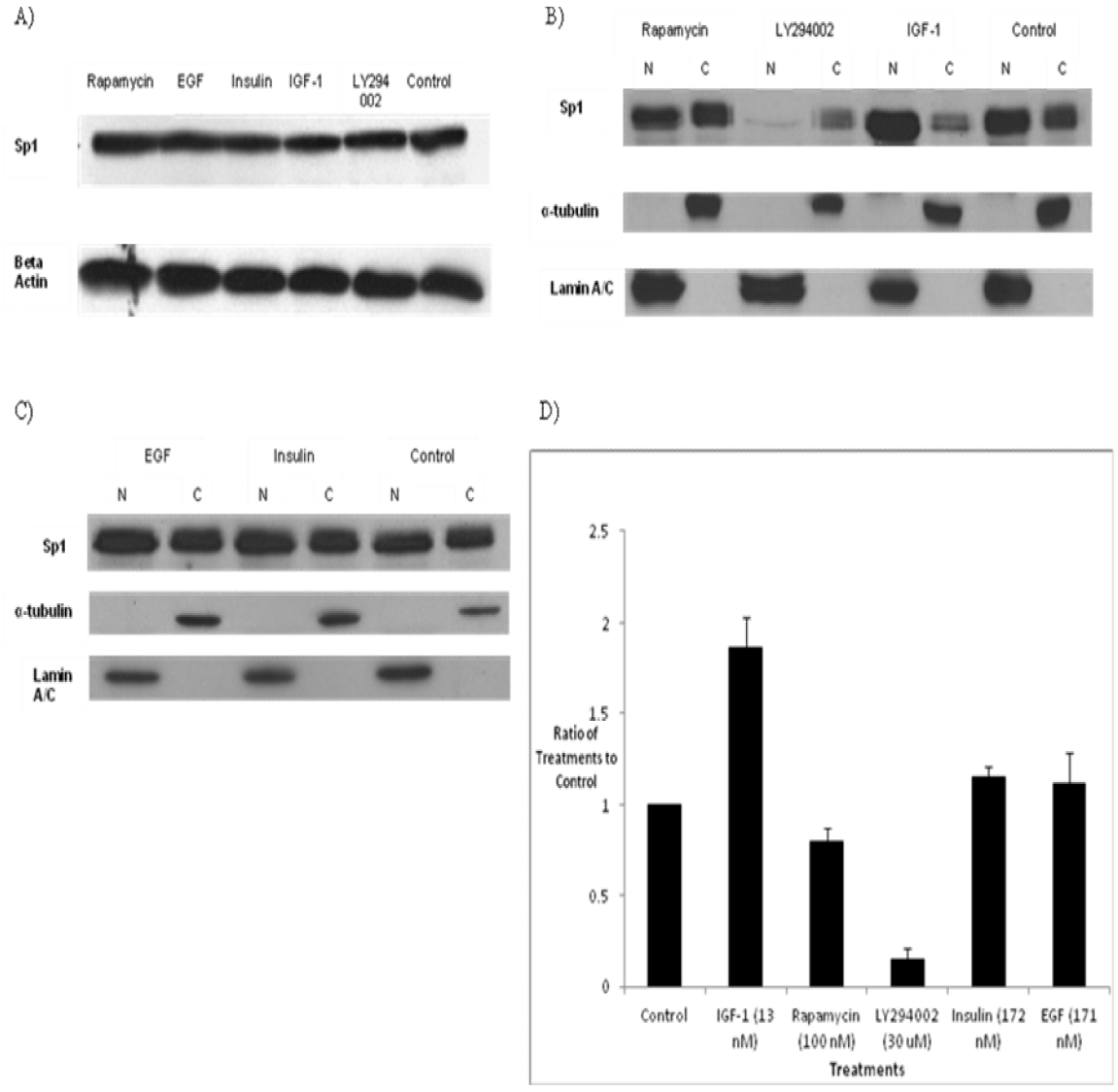


Figure 2.5: Effect of PI3K/AKT/mTOR pathway modulators on Sp1 protein nuclear and cytoplasmic abundance. A) Effect on total Sp1 protein. Cells were cultured as described in previous figures and treated with appropriate growth factors (IGF-1 at 13 nM, insulin at 172 nM, EGF at 171 nM, LY294002 at 30 uM and rapamycin at 100 nM). Total cell extracts were performed after 72 hours of treatment and were immunoblotted against an anti-Sp1 antibody (PEP-2). Beta-actin is provided as a loading control. B,C) Effect on nuclear Sp1 abundance. Cells were fractionated after 72 hours of treatment into cytoplasmic (C) and nuclear (N) extracts. α-tubulin and lamin A/C are provided as technical controls for cell fractionation. D) Densitometric measurements of Sp1 nuclear abundance. Sp1 nuclear abundance was quantified densitometrically (normalized to lamin A/C) and results are shown as mean ratios of treated cells to untreated control cells. Error bars indicate SEM from two independent experiments.

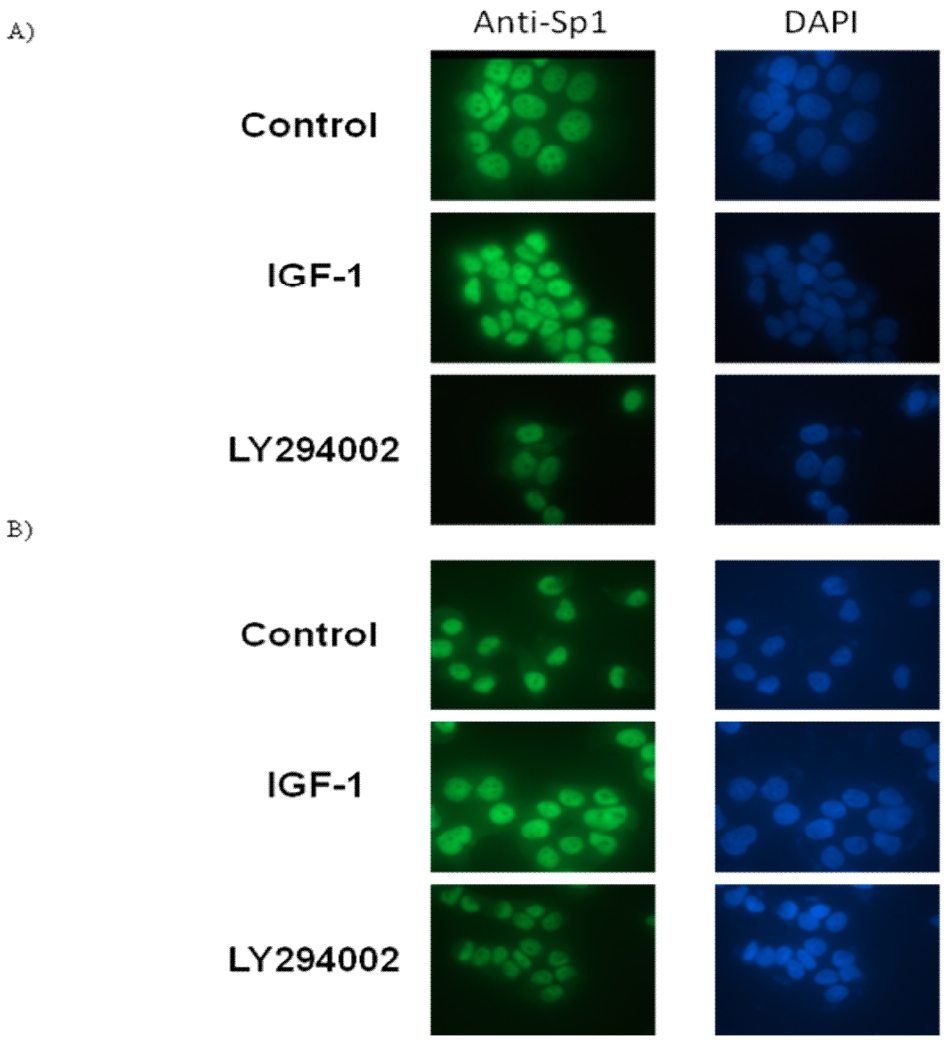


Figure 2.6: Immunofluorescence staining of Sp1. A) MCF-7 cells were seeded at $5x10^4$ cells per well in a Labtech 8-well microscopy slide and left to incubate for 24-hours. Then, the media was changed to serum free with the appropriate treatments (IGF-1 (13nM) or LY294002 (30 uM)) for 72 hours before immunostaining. DAPI staining indicates nuclei. B) Same as A with the T47D cell line.

IGF-1, Rapamycin, LY294002 lead to changes in Sp1 posttanslational modifications in MCF-7 cells

In order to understand the regulation of Sp1 nuclear abundance by the different treatments examined so far, we investigated their effect on five posttranslational modifications of Sp1 known to possibly affect its nuclear abundance (39): levels of total serine, tyrosine, threonine phosphorylation, SUMOylation and O-linked-N-acetylglucosamination (O-GlcNAcylation). Levels of O-GlcNAcylation of Sp1 were most increased in rapamycin (100nM) treated cells and LY294002 (30uM) treated cells, approximately 350% and 200% of control. Conversely, IGF-1 (13nM) treated cells exhibited less Sp1 O-GlcNAcylation (about 60% of control). Insulin and EGF had no significant effect on Sp1 O-GlcNAcylation. Furthermore, total levels of phospho-serine Sp1 were higher in IGF-1 treated cells, about 140% of control, but less in rapamycin treated cells, about 70% of control. LY294002, EGF, Insulin had no effect on Sp1 total serine phosphorylation. Finally, none of the treatments induced a significant change in total Sp1 tyrosine and threonine phosphorylation nor on total Sp1 SUMOylation(Figure 2.7 A,B).

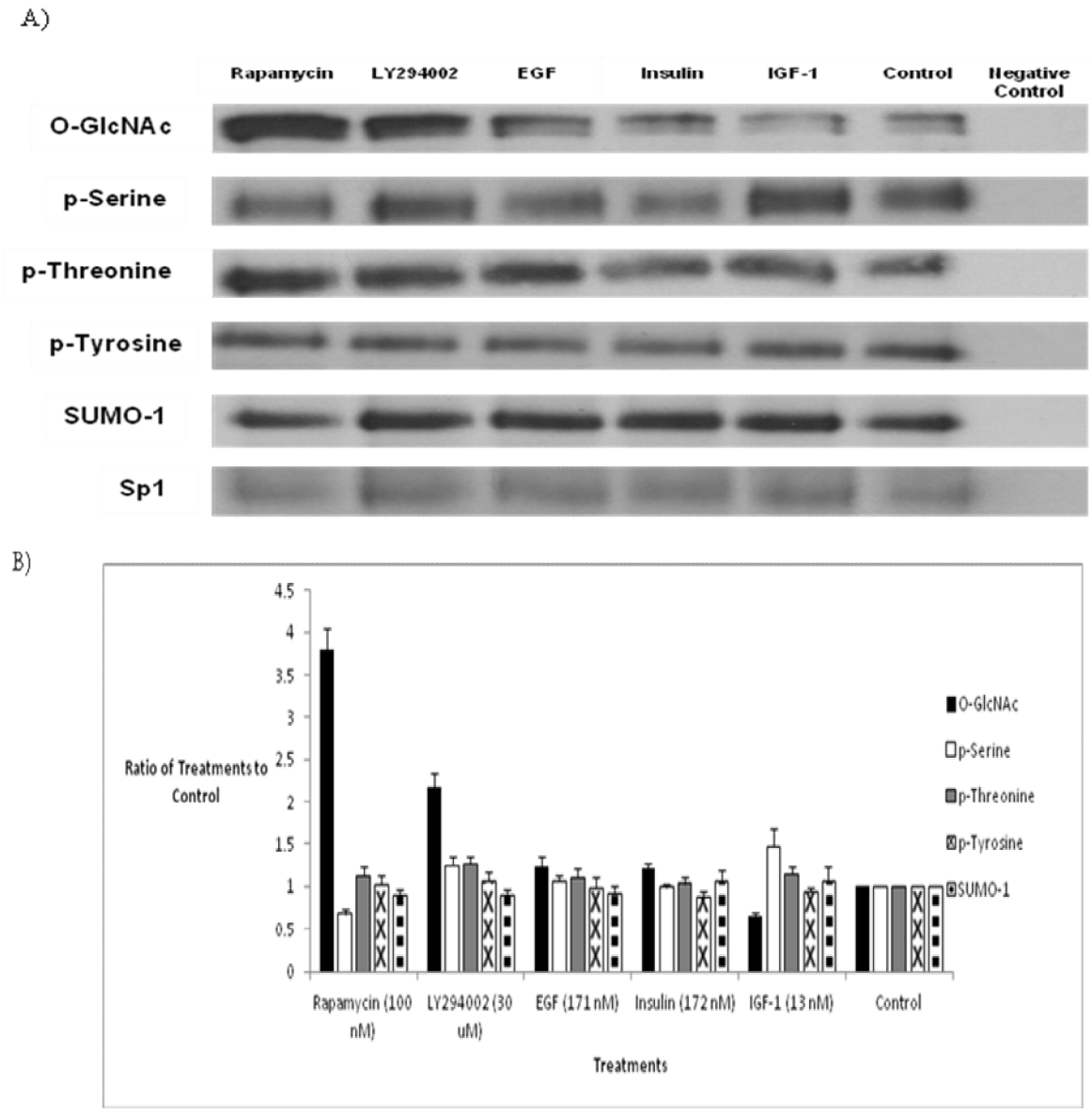


Figure 2.7: Effect of PI3K/AKT/mTOR pathway modulators on Sp1 posttranslational modifications. A) Cells were cultured in 10% FBS media in 10mm dishes until 70% confluence was reached, then treated for 72 hours in serum-free media containing the appropriate compounds. Cells were then lysed in RIPA buffer containing protease and phosphatase inhibitors, and Sp1 was immunoprecipitated and then immunoblotted with different probing antibodies against phospho-serine, phospho-tyrosine, phospho-threonine, SUMO, O-GlcNAc. Then, membranes were stripped and immunoblotted with an antibody against Sp1 to provide a normalization reference (one blot is shown as example). The lane termed negative control is a lysate from control untreated cells where no anti-Sp1 antibody was added. B) Densitometric measurements of Sp1 posttranstlational modifications. Each Sp1 posttranslational modification was quantified and normalized to its corresponding total Sp1 measurement. Results are expressed as mean ratios of treatments to control and the error bars represent the SEM from two different independent experiments.

Effect of IGF-1, insulin and EGF result on activation of AKT vary with time

IGF-1, insulin and EGF had different effects on Sp1 localization and IGFBP-2 mRNA abundance (Figures 2.1, 2.5). To explore the possibility that this was attributable to different degrees of AKT activation, we measured phospho-serine 473 AKT levels under each treatment. Figure 2.8 A and B show that IGF-1 (13 nM) is the most potent activator of AKT after 15 minutes, about 50% more potent than insulin (172 nM) and EGF (171 nM). Also, Figure 2.8A shows, as expected, inhibition of AKT by LY294002 (30 uM) and inhibition of p70 S6 Kinase by rapamycin (100 nM), confirming the known actions of these compounds. After 72 hours however, IGF-1 treated cells showed much more increased phospho-serine 473 AKT levels compared to insulin or EGF treated cells (Figure 2.8 C).

It has previously been shown that IGF-1 increases IGFBP-2 protein expression in an IGF-1 receptor and PI3K dependent fashion (35), as inhibitors of IGF-1 receptor (AG1024) and of PI3K (LY294002) reverse the effects of IGF-1 on IGFBP-2 protein expression but inhibitors of p38 MAPK (SB203580) or p44/p42 MAPK/ERK1/2 (PD98059) do not. We confirm this pattern is also seen for the IGFBP-2 proximal promoter driven luciferase expression. The 200bp sequence construct transfected cells exhibit, as previously shown, an upregulation in luciferase expression when treated with IGF-1 (13 nM), but this effect is abrogated when cells are treated with AG1024 (10 uM) or LY294002 (30 uM). On the other hand, PD98059 (30 uM) and SB203580 (10 uM) have no effect on IGF-1 induced expression of luciferase. Thus, IGF-1 increases the 200bp-driven luciferase expression in the same IGF1-R and PI3K dependent fashion (Figure 2.9).

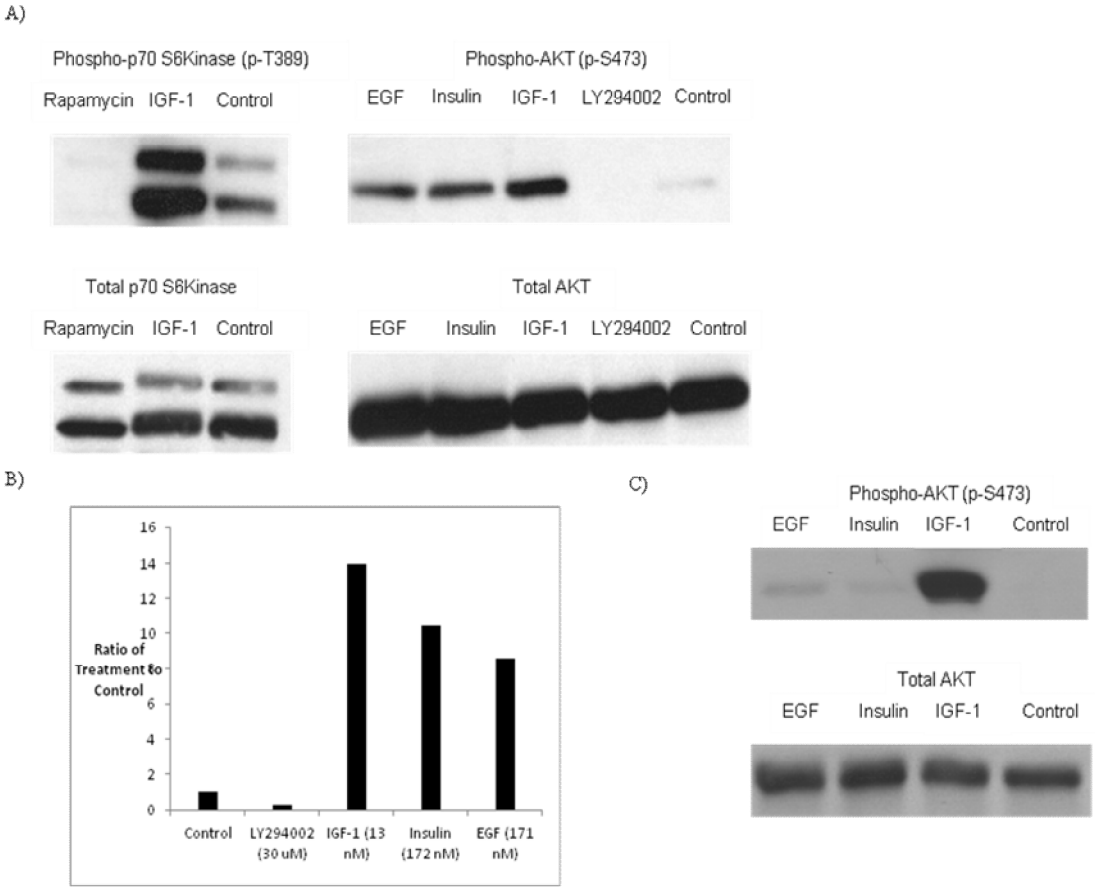


Figure 2.8: Signaling of PI3K/AKT/mTOR modulators. A) Cells were seeded at $5x10^5$ cells per well in 6-well plates, left to incubate for 24 hours and transferred to serum free conditions for an additional 24 hours. Media was replaced with appropriate IGF-1 (13 nM), EGF (171 nM), insulin (172 nM) containing media and cells were lysed after 15 minutes. For inhibitors LY294002 (30 uM) and rapamycin (100 nM), cells were incubated in SFM media for 3 hours before an IGF-1 (13 nM) stimulation for 15 min. Phospho-AKT, Phospho-p70 S6Kinase, total AKT and p70 S6Kinase were the signaling end-points analyzed using Western Blotting. B) Densitometric quantification of the effect on phospho-AKT. Phospho-AKT was densitometrically quantified (normalized to total AKT) and measurements were divided by control. C) Same as A with treatments IGF-1 (13 nM), EGF (171 nM), insulin (172 nM) and 72 hours cultured cells.

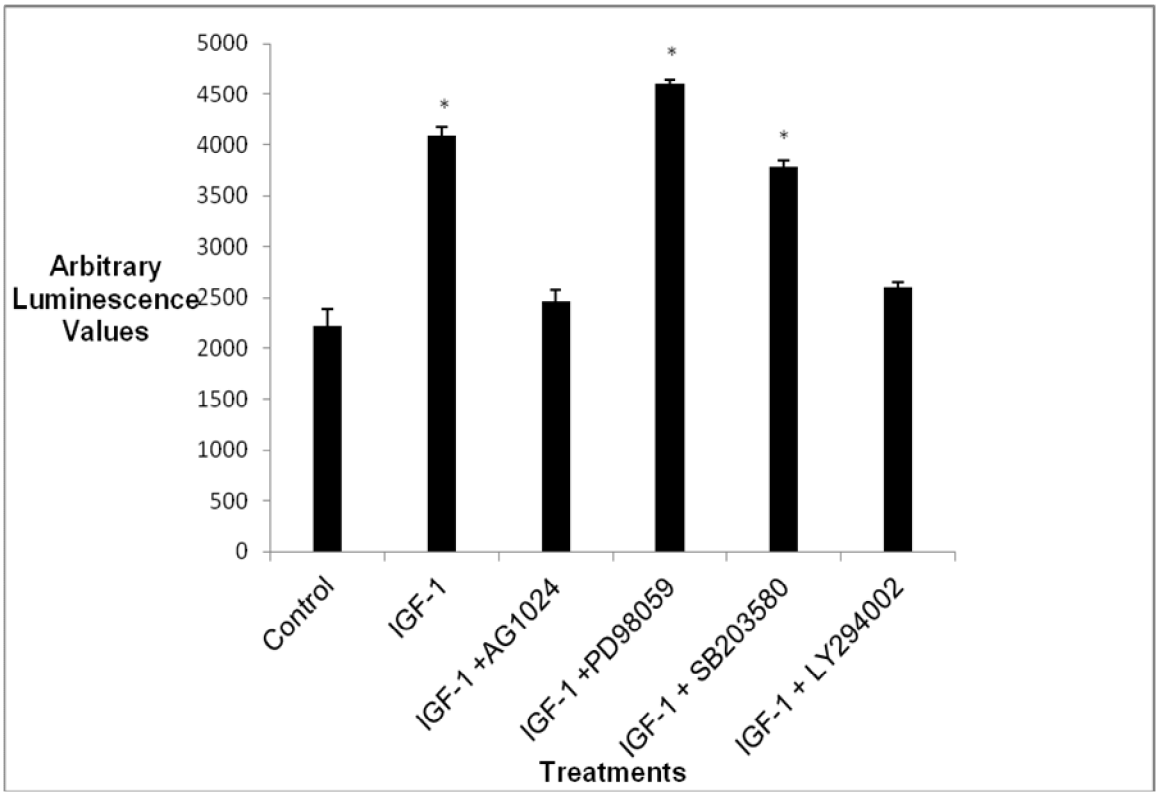


Figure 2.9: IGF-1 activation of the 200bp construct. $4x10^5$ cells were transfected with 2ug of 200bp construct. 24-hours later media was changed to serum free control or IGF-1 (13 nM) or IGF-1 and the following AG1024 (10 uM), LY294002 (30 uM), PD98059 (30 uM), SB203580 (10uM). 72 hours later changes in luciferase expression were measured. Error bars are SEM and * indicates statistical significance compared to control as obtained by ANOVA (n=6 (in duplicate), p-value < 0.00001)

2.5 Discussion

Although regulation of IGFBP-2 protein levels has been associated with the PI3K/AKT/mTOR pathway, an important gap in knowledge existed with respect to the mechanism by which this regulation is achieved. We report here that the changes previously observed by others at the protein level are due mainly to regulation of IGFBP-2 mRNA by this pathway. Specifically, IGF-1 increases IGFBP-2 mRNA abundance while LY294002 and rapamycin decrease it. The observation related to rapamycin is contrary to what might have been anticipated given its well characterized effects on mTOR and protein translation (38). Our luciferase assays demonstrate that this pathway achieves regulation of IGFBP-2 mRNA through a regulatory region present in the first 200 bp upstream of the IGFBP-2 transcription start site. This region has been shown to bind Sp1 transcription factor in the rat. By ectopic overexpression of Sp1, we show that this transcription factor increases expression of luciferase when this 200bp sequence is placed upstream. Additionally, we demonstrate by chromatin immunoprecipitation an increased binding of Sp1 at the IGFBP-2 proximal promoter region in IGF-1 treated cells and a decreased binding in rapamycin and LY294002 treated cells. We show that manipulation of the PI3K/AKT/mTOR pathway does not induce any change in total Sp1, but alters nuclear abundance of the transcription factor in the same direction and at a comparable magnitude as the changes induced in IGFBP-2 protein levels. Furthermore, we demonstrate that levels of O-GlcNAcylation of Sp1 correlate negatively with its nuclear abundance and that total levels of phospho-serine Sp1 partially correlate with its nuclear abundance. We also demonstrate that IGF-1 induces an increase in IGFBP-2 proximal promoter driven luciferase in a PI3K and IGF-1 receptor dependent manner. This provides a mechanistic explanation for the observed effects of the PI3K/AKT/mTOR pathway on IGFBP-2 protein levels.

Our experiments have provided an example of an unusual effect of rapamycin, a well-known inhibitor of translation (38), on IGFBP-2 gene transcription. We show

that in MCF-7 cells, inhibition of mTOR by rapamycin leads to a decrease in nuclear Sp1 levels, but not in total Sp1 levels. Similar observations have been reported in yeast where mTOR regulates nutrient metabolism by sequestration of specific transcription factors in the cytoplasm (40,41). In humans, mTOR regulation of gene transcription has only been observed with respect to ribosomal genes and rRNA. More precisely, it has been shown that the activity and cellular compartamentalization of transcription factor TIF-1A, whose involvement in RNA Pol I transcription is crucial, is modulated by mTOR. This regulation is achieved by a complex balance of kinases and phosphatases modulating two oppositely acting phosphorylation sites on TIF-1A (42). Although mTOR does not phosphorylate TIF-1A directly, protein phosphatase 2A (PP2A), a target of mTOR whose activity increases quickly after rapamycin treatment, has been shown to alter phosphorylation level and cellular localization of TIF-1A (42). In the case of Sp1, we have shown that rapamycin treatment increases O-GlcNAc levels and decreases total phospho-serine levels of the transcription factor, two posttranslational modifications reported to influence Sp1 nuclear abundance (39). The molecular mechanisms underlying these posttranslational modifications and the precise residues of Sp1 involved remain however unknown.

Majumdar *et al*. (43) showed that insulin induced nuclear accumulation and reciprocal O-GlcNAcylation and phosphorylation on serine residues of Sp1 in H-411E liver cells. They reported that serine O-GlcNAcylation accumulates rapidly on Sp1 after treatment and declines thereafter as it is replaced by phosphorylation and as Sp1 total nuclear levels rise. Consistently, our results show that IGF-1 treated cells (which exhibited the highest nuclear Sp1 level) displayed a decrease in Sp1 O-GlcNAcylation and an increase in Sp1 serine phosphorylation. Rapamycin treated cells, which displayed lower total nuclear Sp1 levels, showed increased O-GlcNAcylation and decreased serine phosphorylation of Sp1. Surprisingly, LY294002 treated cells, which induced the most dramatic decline in nuclear Sp1 levels, displayed increased Sp1 O-GlcNAcylation but no corresponding decrease of Sp1 serine phosphorylation,

which suggests a different mechanism of action of LY294002 on Sp1 nuclear abundance. Contrary to Majumdar *et al*., we report no change in Sp1 nuclear abundance with insulin treatment, an observation which is likely due to the non-hepatic nature of the cell lines in our study. Although we report a correlation between increased nuclear Sp1abundance and decreased Sp1 O-GlcNAcylation, and a partial correlation between an increase in total phospho-serine Sp1 and nuclear abundance, the mechanisms behind these observations remain unknown. As Sp1 contains more than 50 putative glycosylation and phosphorylation sites (44), it is unclear which residues are involved in the observed changes in serine phosphorylation and O-GlcNacylation. Elucidation of the mechanism of Sp1 activation in the context of IGFBP-2 transcriptional modulation is the subject of ongoing work in our laboratory.

We also observed that although activation of the PI3K/AKT/mTOR pathway by IGF-1, insulin and EGF led to comparable levels of phospho-serine 473 AKT in the short term (15 min), it was not the case in the long term (3 days). Indeed, IGF-1 was a much more potent activator of AKT after 72 hours of treatment than both insulin and EGF. This difference is the probable cause of the observed discrepancies in IGFBP-2 mRNA/protein abundance and Sp1 posttranslational modifications and nuclear levels between IGF-1 treated cells and insulin or EGF treated cells.

Finally, as Sp1 is involved in the regulation of IGFBP-2 and as there is evidence that IGFBP-2 overexpression can lead to more aggressive cancer behavior by potentiating IGF-1 actions (17-22), our results motivate *in vivo* and translational research to determine if clinical activity of Sp1 inhibitors such as Mithramycin A (which has been used in the treatment of some cancers (45,46)) can be correlated with its effects on Sp1 and/or IGFBP-2. It is conceivable that Sp1 inhibitors will be particularly useful in cancers overexpressing IGFBP-2.

Chapter References

1. Firth SM, Baxter RC 2002 Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev 23:824-854

2. Hwa V, Oh Y, Rosenfeld RG 1999 The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocr Rev 20 :761-787

3. Drop SLS, Schuller AGP, Lindenbergh-Kortleve DJ, Groffen C, Brinkman A, Zwarthoff EC 1992 Structural aspects of the IGFBP family. Growth Regulation 2:69-79

4. Baxter R 1993 Circulating binding proteins for the insulin-like growth factors. Trends Endocrinol Metab 4:91-96

5. Jones JI, Clemmons DR 1995 Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev 16:3-34

6. Chesik D, De Keyser J, Wilczak N 2007 Insulin-like growth factor binding protein-2 as a regulator of IGF actions in CNS: implications in multiple sclerosis. Cytokine Growth Factor Rev 18:267-278

7. Campbell PG, Durham SK, Hayes JD, Suwanichkul A, Powell DR 1999 Insulin-like growth factor-binding protein-3 binds fibrinogen and fibrin. J Biol Chem 274:30215-30221

8. Parker A, Rees C, Clarke J, Busby WH, Jr., Clemmons DR 1998 Binding of insulin-like growth factor (IGF)-binding protein-5 to smooth-muscle cell extracellular matrix is a major determinant of the cellular response to IGF-I. Mol Biol Cell 9:2383-2392

9. Hoflich A, Lahm H, Blum W, Kolb H, Wolf E 1998 Insulin-like growth factor-binding protein-2 inhibits proliferation of human embryonic kidney fibroblasts and of IGF-responsive colon carcinoma cell lines. FEBS Lett 434:329-334

10. Mohan S, Baylink DJ 2002 IGF-binding proteins are multifunctional and act via IGF-dependent and -independent mechanisms. J Endocrinol 175:19-31

11. Frommer KW, Reichenmiller K, Schutt BS, Hoeflich A, Ranke MB, Dodt G, Elmlinger MW 2006 IGF-independent effects of IGFBP-2 on the human breast cancer cell line Hs578T. J Mol Endocrinol 37:13-23

12. Perks CM, Vernon EG, Rosendahl AH, Tonge D, Holly JM 2007 IGF-II and IGFBP-2 differentially regulate PTEN in human breast cancer cells. Oncogene 26:5966-5972

13. Okabe E, Kajihara J, Usami Y, Hirano K 1999 The cleavage site specificity of human prostate specific antigen for insulin-like growth factor binding protein-3. FEBS Lett 447:87-90

14. Yu J, Iwashita M, Kudo Y, Takeda Y 1998 Phosphorylated insulin-like growth factor (IGF)-binding protein-1 (IGFBP-1) inhibits while non-phosphorylated IGFBP-1 stimulates

IGF-I-induced amino acid uptake by cultured trophoblast cells. Growth Horm IGF Res 8:65-70

15. Sommer A, Spratt SK, Tatsuno GP, Tressel T, Lee R, Maack CA 1993 Properties of glycosylated and non-glycosylated human recombinant IGF binding protein-3 (IGFBP-3). Growth Regul 3:46-49

16. Lalou C, Lassarre C, Binoux M 1996 Isolation and characterization of proteolytic fragments of insulin-like growth factor-binding protein-3. Horm Res 45:156-159

17. Chen JC, Shao ZM, Sheikh MS, Hussain A, LeRoith D, Roberts CT, Fontana JA 1994 Insulin-like growth factor-binding protein enhancement of insulin-like growth factor-1 (IGF-1)-mediated DNA synthesis and IGF-1 binding in a human breast carcinoma cell line. J Cell Physiol 158:69-78

18. Hoeflich A, Fettscher O, Lahm H, Blum WF, Kolb HJ, Engelhardt D, Wolf E, Weber MM 2000 Overexpression of insulin-like growth factor-binding protein-2 results in increased tumorigenic potential in Y-1 adrenocortical tumor cells. Cancer Res 60:834-838

19. Menouny M, Binoux M, Babajko S 1998 IGFBP-2 expression in a human cell line is associated with increased IGFBP-3 proteolysis, decreased IGFBP-1 expression and increased tumorigenicity. Int J Cancer 77:874-879

20. Dunlap SM, Celestino J, Wang H, Jiang R, Holland EC, Fuller GN, Zhang W 2007 Insulin-like growth factor binding protein 2 promotes glioma development and progression. Proc Natl Acad Sci U S A 104:11736-11741

21. So A, Levitt RJ, Eigl B, Fazil L, Muramaki M, Leung S, Cheang M, Nielsen T, Gleave M, Pollak M 2008 Insulin-like Growth Factor Binding Protein-2 is a Novel Therapeutic Target Associated with Breast Cancer. Clin Cancer Res 14:6944-6954

22. Hoeflich A, Reisinger R, Lahm H, Kiess W, Blum WF, Kolb HJ, Weber MM, Olf E 2001 Insulin-like growth factor-binding protein 2 in tumorigenesis: protector or promoter? Cancer Res 61:8601-8610

23. Boulle N, Logie A, Gicquel C, Perin L, Le Bouc Y 1998 Increased levels of insulin-like growth factor II (IGF-II) and IGF-binding protein-2 are associated with malignancy in sporadic adrenocortical tumors. J Clin Endocrinol Metab 83:1713-1720

24. McDonald KL, O'Sullivan MG, Parkinson JF, Shaw JM, Payne CA, Brewer JM, Young L, Reader DJ, Wheeler HT, Cook RJ, Biggs MT, Little NS, Teo C, Stone G, Robinson BG 2007 IQGAP1 and IGFBP2: valuable biomarkers for determining prognosis in glioma patients. J Neuropathol Exp Neurol 66:405-417

25. Busund LT, Richardsen E, Busund R, Ukkonen T, Bjornsen T, Busch C, Stalsberg H 2005 Significant expression of IGFBP2 in breast cancer compared with benign lesions. J Clin Pathol 58:361-366

26. Richardsen E, Ukkonen T, Bjornsen T, Mortensen E, Egevad L, Busch C 2003 Overexpression of IGBFB2 is a marker for malignant transformation in prostate epithelium. Virchows Arch 442:329-335

27. Zumkeller W, Schwander J, Mitchell CD, Morrell DJ, Schofield PN, Preece MA 1993 Insulin-like growth factor (IGF)-I, -II and IGF binding protein-2 (IGFBP-2) in the plasma of children with wilms' tumor. Eur J Cancer 29A:1973-1977

28. Fottner C, Sattarova S, Hoffmann K, Spottl G, Weber MM 2008 Elevated serum levels of IGF-binding protein 2 in patients with non-seminomatous germ cell cancer: correlation with tumor markers alpha-fetoprotein and human chorionic gonadotropin. Eur J Endocrinol 159:317-327

29. Han HJ, Kang CW, Park SH 2006 Tissue-specific regulation of insulin-like growth factors and insulin-like growth factor binding proteins in male diabetic rats in vivo and in vitro. Clin Exp Pharmacol Physiol 33:1172-1179

30. Ernst CW, White ME 1996 Hormonal regulation of IGF-binding protein-2 expression in proliferating C2C12 myoblasts. J Endocrinol 149:417-429

31. Chan TW, Pollak M, Huynh H 2001 Inhibition of insulin-like growth factor signaling pathways in mammary gland by pure antiestrogen ICI 182,780. Clinical Cancer Research 7:2545-2554

32. Elminger MW, Bell M, Schuett BS, Langkamp M, Kutoh E, Ranke MB 2001 Transactivation of the IGFBP-2 promoter in human tumor cell lines. Mol Cell Endocrinol 175:211-218

33. Blume-Jensen P, Hunter T 2001 Oncogenic kinase signalling. Nature 411 :355-365

34. Levitt RJ, Georgescu MM, Pollak M 2005 PTEN-induction in U251 glioma cells decreases the expression of insulin-like growth factor binding protein-2. Biochem Biophys Res Commun 336:1056-1061

35. Martin JL, Baxter RC 2007 Expression of insulin-like growth factor binding protein-2 by MCF-7 breast cancer cells is regulated through the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin pathway. Endocrinol 148:2532-2541

36. Courey AJ, Tjian R 1988 Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. Cell 55:887-898

37. Boisclair YR, Brown AL, Casola S, Rechler MM 1993 Three clustered Sp1 sites are required for efficient transcription of the TATA-less promoter of the gene for insulin-like growth factor-binding protein-2 from the rat. J Biol Chem 268:24892-24901

38. Gingras AC, Raught B, Sonenberg N 2004 mTOR signaling to translation. Curr Top Microbiol Immunol 279:169-197

39. Solomon SS, Majumdar G, Martinez-Hernandez A, Raghow R 2008 A critical role of Sp1 transcription factor in regulating gene expression in response to insulin and other hormones. Life Sci 83:305-312

40. Hardwick JS, Kuruvilla FG, Tong JK, Shamji AF, Schreiber SL 1999 Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins. Proc Natl Acad Sci U S A 96:14866-14870

41. Beck T, Hall MN 1999 The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. Nature 402:689-692

42. Mayer C, Zhao J, Yuan X, Grummt I 2004 mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability. Genes Dev 18:423-434

43. Majumdar G, Harrington A, Hungerford J, Martinez-Hernandez A, Gerling IC, Raghow R, Solomon S 2006 Insulin dynamically regulates calmodulin gene expression by sequential o-glycosylation and phosphorylation of sp1 and its subcellular compartmentalization in liver cells. J Biol Chem 281:3642-3650

44. Tan NY, Khachigian LM 2009 Sp1 phosphorylation and its regulation of gene transcription. Mol Cell Biol 29:2483-2488

45. Dutcher JP, Coletti D, Paietta E, Wiernik PH 1997 A pilot study of alpha-interferon and plicamycin for accelerated phase of chronic myeloid leukemia. Leuk Res 21:375-380

46. Kennedy BJ, Torkelson JL 1995 Long-term follow-up of stage III testicular carcinoma treated with mithramycin (plicamycin). Med Pediatr Oncol 24:327-328

Chapter 3

Binding between IGF-1 and IGFBP-3 is not influenced by glucose or 2-DG

This section is based on the following published article:

Mireuta M, Hancock MA, Pollak M. Binding between insulin-like growth factor 1 and insulin-like growth factor binding protein 3 is not influenced by glucose or 2-deoxy-D-glucose. *J Biol Chem* 2011 May 13;286(19):16567-73. Epub 2011 Mar 9.

Preface

For the second part of the thesis, we examine aspects of IGF-1:IGFBP-3 complex formation in the context of small binding inhibitors. As previously described in the literature review, the classical role of IGFBPs is to regulate IGF bioavailability. Therefore, there is some interest in small molecules interfering with IGF:IGFBP complex formation. A recent report suggested that 2-deoxyglucose (2-DG), a well-known inhibitor of glycolysis, unexpectedly disrupts IGF-1:IGFBP3 complex formation and upregulates IGF-1R signaling in various cell lines. This report, if true, would have major implications in both IGF physiology and the use of 2-DG as a potential therapeutic agent in cancer. Additionally, it was plausible that glucose, because of its related structure, could have similar effects. The latter finding would have had tremendous implications in the fields of IGF physiology and metabolism.

Therefore, we investigated the effects of 2-DG and glucose on IGF-1:IGFBP-3 binding. We initially wanted to further characterize the effect of these small molecules, particularly by examining the induced changes in affinity constants. Unfortunately, we were unable to reproduce the results of the original article.

3.1 Abstract

A recent report details that 2-deoxy-D-glucose (2-DG), a well known inhibitor of glycolysis and a candidate antineoplastic agent, also induces insulin-like growth factor 1 receptor (IGF-1R) signaling through the inhibition of insulin-like growth factor 1/ insulin-like growth factor binding protein 3 (IGF-1:IGFBP-3) complex formation. The authors hypothesized that disrupted IGF-1:IGFBP-3 binding by 2-DG led to increased free IGF-1 concentrations and, consequently, activation of IGF-1R downstream pathways. Since their report suggests unprecedented off-target effects of 2-DG, this has profound implications for the fields of metabolism and oncology. Using ELISA, surface plasmon resonance (SPR), and novel "intensity-fading" mass spectrometry (MS), we now provide a detailed characterization of complex formation between IGF-1 and IGFBP-3. All three of these independent methods demonstrated that there was no effect of glucose or 2-DG on the interaction between IGF-1 and IGFBP-3. Furthermore, we show examples of 2-DG exposure associated with reduced rather than increased IGF-1R and AKT activation, providing further evidence against a 2-DG increase in IGF-1R activation by IGF-1:IGFBP-3 complex disruption.

3.2 Introduction

Insulin-like Growth Factors I and II (IGF-1, IGF-2) are peptide hormones similar in molecular structure to insulin and they regulate a variety of cellular activities including metabolism, proliferation and growth. Both IGFs bind to the IGF-1 receptor (IGF-1R) at the cell membrane and initiate a signaling cascade that results in the activation of the PI3K/AKT/mTOR (Phosphadityl Inositol 3 Kinase/AKT/ mammalian Target Of Rapamycin) pathway (1-5). The IGF-2 receptor (IGF-2 R), which binds to IGF-2, lacks an intracellular signaling domain and is therefore considered to only act as a sink for excess IGF-2 (6).

IGF actions are tightly modulated by a family of proteins called Insulin-like Growth Factor Binding Proteins (IGFBPs), of which IGFBP-1 through 6 have been characterized. Most IGFBPs in the blood originate from the liver, but they are also expressed in many other tissues. IGFBP-3 and 5 are the most abundant IGFBPs in the circulation. They form a ternary complex with IGFs and a third protein termed Acid-Labile Subunit (ALS) (7-9). IGFBPs are known to modulate actions of IGFs both in vitro and in vivo (7). Since only free IGFs are considered ligands for the IGF-1R, significant research efforts have focused on small molecules capable of interfering with IGF-IGFBP binding.

A recent report showed that 2-deoxy-D-glucose (2-DG), a close derivative of glucose, promoted dissociation of IGF-1 from IGFBP-3 and consequently contributed to elevated phosphoserine 473 AKT levels in a variety of cancer cell lines (10). 2-DG has been proposed as a potential therapeutic agent in the treatment of cancer since it interferes with glycolysis (11). Entering the cell through glucose transporters, 2-DG inhibits enzymes of the glycolytic pathway both competitively (phosphoglucose isomerase) (12) and non-competitively (hexokinase)(13). Since tumor cells depend more heavily on glycolysis compared to normal cells, 2-DG is under investigation for cancer treatment (14).

The recent study by Zhong and co-workers (10) showed that 2-DG, apart from its classic activities as an inhibitor of glycolysis, can also increase IGF-1R signaling by disrupting IGF-1:IGFBP-3 binding. As increased IGF-1R signaling is associated with greater proliferation of tumor cells, this report has shed doubt on the efficacy of 2-DG as a cancer therapeutic. To examine this issue further, the present study characterizes the effect of glucose and 2-DG on binding between IGF-1 and IGFBP-3. Using ELISA, Surface Plasmon Resonance (SPR), and novel “intensity-fading” Mass Spectrometry (MS), we report that glucose and 2-DG have no effect on IGF-1:IGFBP-3 complex formation.

3.3 Materials and Methods

Materials

Human recombinant IGF-1 was purchased from Feldan Biosciences (Montreal, Canada) and human recombinant IGFBP-3 was obtained from Insmed Incorporated (Glen Allen, Virginia, USA). Glucose, 2-DG, and fatty-acid free bovine serum albumin (BSA) were purchased from Sigma (Mississauga, Ontario, Canada). MCF-7, T47D and HeLa cell lines were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured in standard DMEM medium supplemented with 10% fetal bovine serum (FBS) and 20 µg/ml Gentamycin. Phosphate buffered saline (PBS; 10 mM Sodium Phosphate, 2.7 mM KCl, 137 mM NaCl) and Hepes buffered saline (HBS-EP; 10 mM Hepes pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) Tween-20) were prepared using analytical grade chemicals.

ELISA

Microtitre plates (Costar, Lowell, MA, USA) were coated with either human recombinant IGF-1 (5µg/ml) or human recombinant IGFBP-3 (2µg/ml) in PBS overnight at room temperature. Plates were washed three times with sample buffer (PBS or HBS-EP; 100µl/well) and then blocked for 3 hours at room temperature using 5% (w/v) casein (in sample buffer; 300µl/well). Plates were washed again in the similar manner before 30 min incubations with IGFBP-3 or 2.5 hour incubations with IGF-1 at room temperature. After washing, primary antibodies (anti-IGFBP-3 or anti IGF-1; Santa Cruz, Santa Cruz, California,USA) were added to each well (100µl of 1:50 dilution in sample buffer containing 5% (w/v) casein) for 1 hour at room temperature. After washing, secondary antibody (Santa Cruz, Santa Cruz, California,USA) was added to each well in the similar manner. After washing, the color developing solution (RnD Systems, Mineapolis, MN, USA) was added to the wells and absorbance readings were monitored at 450 nm.

SPR

Label-free, real-time binding between IGF-1 (7.6 kDa) and IGFBP-3 (29 kDa) was examined using a Biacore 3000 system (GE Healthcare Bio-Sciences, AB, USA) at 25°C with filtered (0.2 µm) and degassed HBS-EP running buffer (10mM HEPES pH 7.4, 150mM NaCl, 3mM EDTA, 0.005% (v/v) Tween20). IGFBP-3 (9 µg/mL in 10 mM sodium acetate pH 5.5) was immobilized to CM4 sensor chips using the Biacore Amine Coupling Kit (~1000 RU final); corresponding reference surfaces were prepared in the absence of IGFBP-3. IGF-1 (0 – 25 nM; or BSA as negative control) was injected over reference and IGFBP-3-immobilized surfaces, in the absence or presence of competitor (25 – 100 mM glucose or 2-DG), using variable flow rates (10-75 µl/min) and contact times (3-5 min association, 5-10 min dissociation). Between sample injections, sensor chip surfaces were regenerated using Pierce Gentle Elution buffer (Thermo Scientific, Illinois, USA) containing 0.1% (v/v) Empigen (Affymetrix-Anatrace, Ohio, USA).

All SPR data was double-referenced (14b) and is representative of duplicate injections acquired from two independent trials. For each titration series, a buffer blank (+/- glucose or 2-DG) was injected first, the highest IGF-1 concentration second, and serial dilutions then followed (from the lowest to the highest concentration repeated). Comparing binding responses between the highest IGF-1 injections verified consistent IGFBP-3-immobilized surface activity throughout each assay. Apparent equilibrium dissociation constants (K_D), as well as individual association (k_a) and dissociation (k_d) rate constants, were determined by global fitting of the data to a “1:1 kinetic” model with or without mass transport (BIAevaluation v4.1 software). The kinetic estimates represent fits to the experimental data where Chi^2 values were <1.5.

Mass Spectrometry

Eppendorf tubes containing IGF-1 (13 nM) or IGF-1 plus IGFBP-3 (13 or 26 nM) samples were incubated for 3 hours on a rocking platform at room temperature. A normal phase (NP-20) protein chip (Bio-Rad, Mississauga, ON, Canada) was

washed three times with 5 µl of HPLC-grade water before the addition of 5 µl of sample per spot and air drying. Subsequently, each spot was washed three times with 5µl of a low stringency buffer (Bio-Rad) and then left to air dry. 5 mg of α-Cyano-4-hydroxycinnamic acid (CHCA; Bio-Rad) was dissolved in 200 µl of solution A (50% (v/v) HPLC-grade water, 49.5% (v/v) acetonitrile, and 0.5% (v/v) trifluoroacetic acid); the mixture was vortexed for 5 minutes and then centrifuged for 10 minutes before the supernatant was collected and further diluted with 200 µl of solution A. The diluted supernatant was then added to each spot (1µl per spot) and left to air dry; this was repeated twice to create two layers. The chip was then analyzed using a Ciphergen Protein Chip Series 4000 instrument (Ciphergen Biosystems, Fremont, CA, USA).

Western Blots

Total cell lysates were obtained using RIPA buffer as described earlier (Chapter 2 material and methods). 50 µg of total protein were loaded per lane and membranes were immunoblotted with the following antibodies phospho-serine 473 AKT, AKT, phospho-tyrosine 1135/1136 IGF-1R beta chain, IGF-1R beta chain and β-actin (Cell Signalling, Danvers, MA, USA).

3.4 Results

Qualitative binding of IGF-1 to IGFBP-3

Initially, ELISA assays were performed in which IGF-1 was immobilized in the microtitre wells (Figure 3.1). In an HBS-based buffer system, saturable, dose-dependent binding of IGFBP-3 was detected and yielded sigmoidal curves (log scale) as expected for typical protein interactions. Binding specificity was evidenced by decreased colorimetric responses in the presence of free IGF-1 (Figure 3.1), as well as high sodium chloride and high Tween-20 detergent concentrations (data not shown). In the presence of excess glucose or 2-DG (25 – 100 mM), however, the binding of IGFBP-3 to immobilized IGF-1 was unaltered in all cases. As negative controls, microtitre wells lacking immobilized protein or primary/secondary antibodies failed to generate any colorimetric response (data not shown). In the reversed orientation, ELISA assays were also performed in which IGFBP-3 was immobilized (Figure 3.2). Saturable, dose-dependent binding of IGF-1 was detected and, similar to above, the interaction could be competed with free IGFBP-3 but was unaltered by glucose or 2-DG. ELISA assays were performed in other buffer settings (PBS, Krebs-Ringer) with identical conclusions (data not shown).

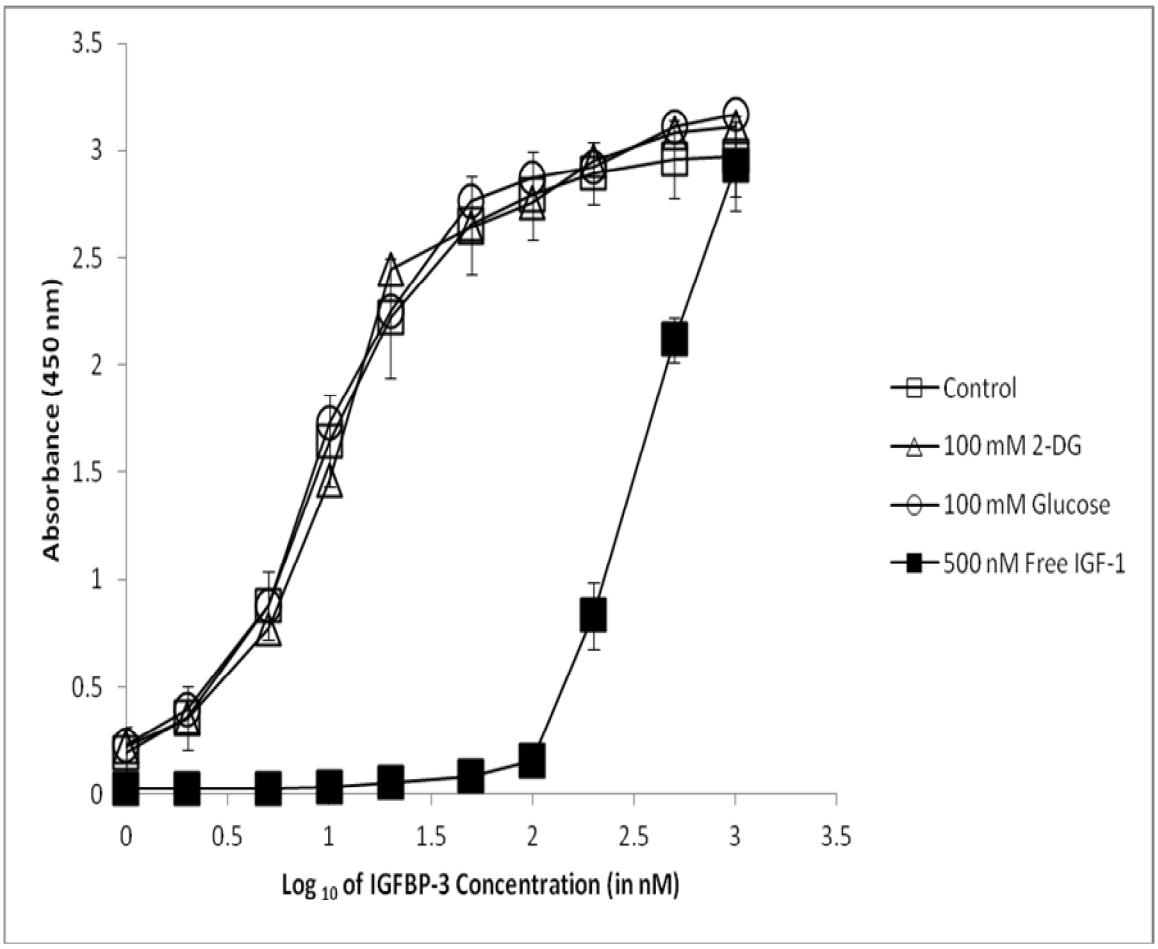


Figure 3.1: ELISA assay to monitor binding of IGFBP3 (0 – 1000 nM in HBS-EP) to immobilized IGF-1 in the absence (open squares) or presence of 100 mM 2-DG (open triangles), 100 mM glucose (open circles), and 500 nM IGF-1 (closed squares). Error bars (standard deviation of triplicate measurements) are only depicted for the "Control" and "500 nM IGF-1" series for simplicity.

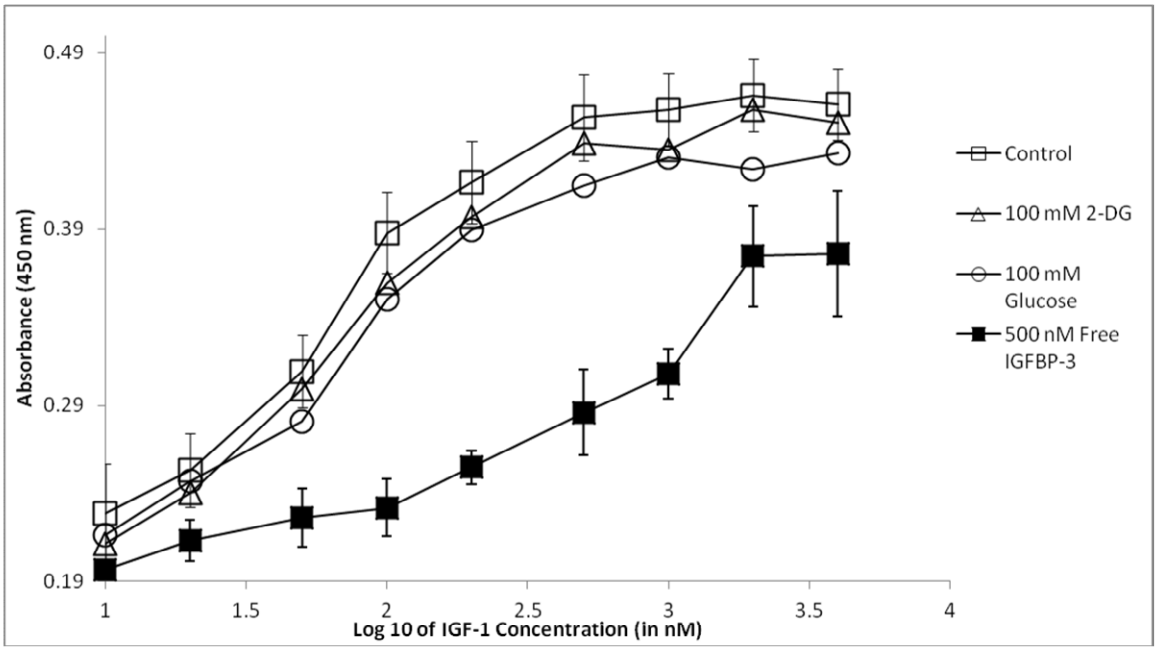


Figure 3.2: Reverse ELISA assay to monitor binding of IGF-1 (0 – 5000 nM in HBS-EP) to immobilized IGFBP-3 in the absence (open squares) or presence of 100 mM 2-DG (open triangles), 100 mM glucose (open circles), and 500 nM IGFBP-3 (closed squares). Error bars (standard deviation of triplicate measurements) are only depicted for the "Control" and "500 nM IGFBP-3" series for simplicity.

Quantitative binding of IGF-1 to IGFBP-3

To validate the ELISA data, binding between IGF-1 and IGFBP-3 was then examined using label-free, real-time SPR and the identical HBS-EP buffer system. A single, low nanomolar concentration of IGF-1 specifically bound to immobilized IGFBP-3 under high flow rate conditions (Figure 3.3), whereas an equimolar concentration of BSA failed to interact (i.e. negative binding control). The overall amounts of IGF-1 bound and the individual association and dissociation kinetics were not significantly altered in the presence of excess glucose or 2-DG (25 – 100 mM). Expanding upon the fixed-concentration specificity tests, IGF-1 was then titrated in the absence and presence of excess 2-DG (Figure 3.4). Visually, the overall dose-dependence and individual kinetics of IGF-1 binding to immobilized IGFBP-3 were not significantly altered in the

presence of excess glucose or 2-DG. Likewise, global fitting of each titration series to a 1:1 kinetic model indicated that there was no significant difference in the sub-nanomolar affinity for this interaction over replicate trials (Table 3.1). In the absence or presence of an additional "k_t" parameter, we also confirmed that the curve fitting was not significantly altered by mass transport limitations (i.e. fitted k_t coefficient was ~10^8 RU M^{-1} s^{-1} as expected).

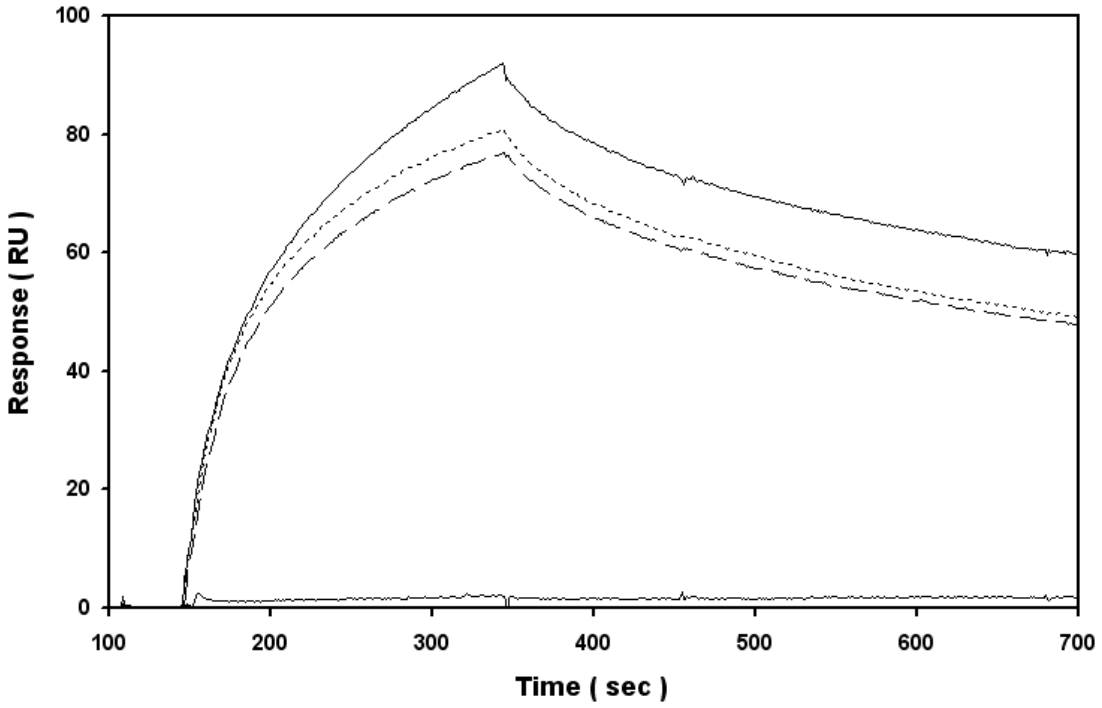


Figure 3.3 : SPR analysis to monitor specificity of IGF-1 (25 nM in HBS-EP) binding to IGFBP-3 (1000 RU amine-coupled) in the absence (upper solid line) or presence of 100 mM 2-DG (dashed line) and 100 mM glucose (dotted line) at 75 µl/min. As a negative binding control, no significant response was observed with 25 nM BSA (bottom solid line). Double-referenced data is representative of duplicate injections acquired from two independent trials.

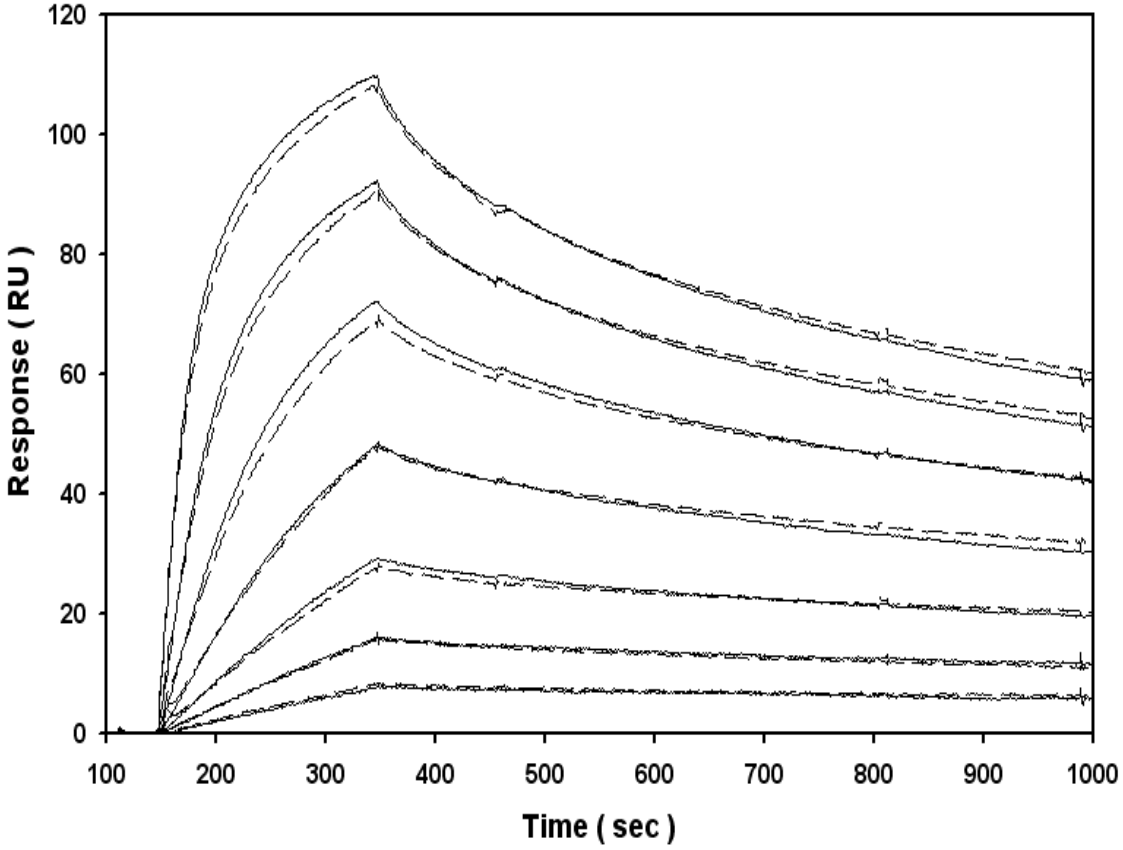


Figure 3.4: SPR analysis to monitor real-time kinetics of IGF-1 (0 – 25 nM in HBS-EP; 2-fold dilution series) binding to IGFBP-3 (1000 RU amine-coupled) in the absence (solid lines) or presence of 100 mM 2-DG (dashed lines) at 75 µl/min (3.5 min association + 10 min dissociation). Double-referenced data is representative of duplicate injections acquired from two independent trials.

100 mM 2-DG	**k_a x 10^6 M^{-1} s^{-1}**	**k_d x 10^{-4} s^{-1}**	**K_D (pM)**
-	**1.40 +/- 0.01**	**8.40 +/- 0.04**	**604 +/- 5**
+	**1.33 +/- 0.01**	**7.93 +/- 0.05**	**599 +/- 5**

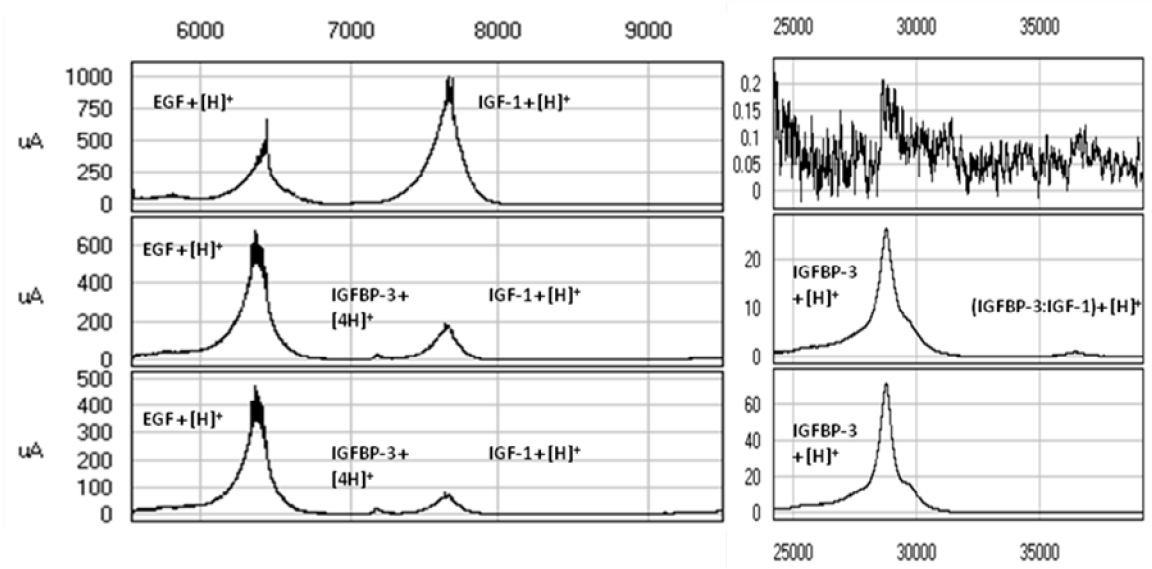
Table 3.1: Apparent kinetics of IGF-1 binding to immobilized IGFBP-3 in the absence and presence of 2-DG, as assessed by SPR. Estimates (+/- standard error of the mean; n=4) represent global analysis of titration series to a "1:1 kinetic" model where goodness-of-fit (Chi^2) values were <1.5; association rate (k_a), dissociation rate (k_d), and equilibrium dissociation (K_D = k_d / k_a) constants were not significantly altered in the presence of an additional mass transport (k_t ~10^8 RU M^{-1} s^{-1}) fitting parameter.

Complex formation between IGF-1 and IGFBP3

To validate the ELISA and SPR data, we also used Mass Spectrometry (MS) to demonstrate that 2-DG and glucose have no effect on IGF-1/ IGFBP-3 binding. In recent years, Surface Enhanced Laser Desorption/Ionization (SELDI) and Matrix Enhanced Laser Desorption/Ionization (MALDI) Time Of Flight (TOF) MS have become a powerful tool for the investigation of non-covalent protein complexes (15). “Intensity Fading” experiments are a relatively novel technique used to directly visualize protein-protein binding without the use of cross-linkers (16). In these experiments, a fixed concentration of protein A (ideally between 5 and 16 kDa) is incubated with different concentrations of its binding partner B; as the concentration of protein B is increased, the intensity of the peak corresponding to protein A slowly diminishes or “fades” (17).

In our MS experiments, we incubated IGF-1 at 13 μM alone, with an equimolar concentration of IGFBP-3 (13 μM), or with excess IGFBP-3 (26 μM) in PBS. Figure 3.5A shows that the intensity of IGF-1 (7600 Da) fades with increasing concentrations of IGFBP-3 (29000 Da), decreasing from 1000 intensity units to under 50 at the highest IGFBP-3 concentration. Epidermal Growth Factor (EGF; 9 μM) was included as an internal, non-binding control and its intensity (6400 Da) remained unaltered across all IGFBP-3 concentrations. As commonly encountered in “intensity-fading” experiments (16, 17), detection of resultant IGF-1:IGFBP-3 complex (36000 Da) was not proportional to theoretical expectations. The same experiments were performed in PBS containing 100mM 2-DG (Figure 3.5B) or 100 mM glucose (data not shown) and similar spectral outcomes were observed. Since neither glucose nor 2-DG was able to reverse the intensity fading of the IGF-1 peak, this assay indicates that they do not influence IGF-1:IGFBP-3 binding.

A)



B)

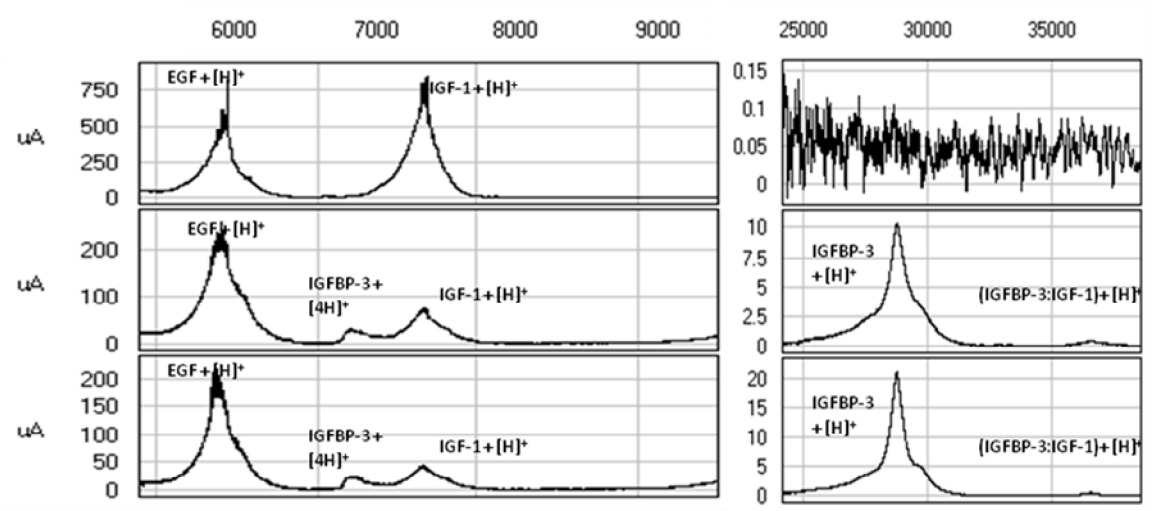


Figure 3.5: MS analysis to monitor binding between IGF-1 (~7,600 Da) and IGFBP-3 (~29,000 Da) in the absence or presence of 100 mM 2-DG (and internal EGF non-binding control, ~6,400 Da). A) Upper boxes represent 13 nM IGF-1 alone, middle boxes represent 13 nM IGF-1 with 13 nM IGFBP-3, and lower boxes represent 13 nM IGF-1 with 26 nM IGFBP-3. B) Identical intensity-fading experiments performed in the presence of 100 mM 2-DG. The data presented is representative of three independent experiments. Y-axes are representative of signal response (uA) and X-axes are representative of molecular mass (Da).

Effects of 2-DG on AKT and IGF-1R

Furthermore, we investigated the effect of 2-DG on IGF-1R and AKT activation in two breast cancer cell lines (MCF-7 and T47D) and one cervical cancer cell line (HeLa). We observed that addition of 2-DG at 25 mM, a concentration previously reported to enhance AKT phosphorylation in these cell lines (21), resulted in a consistent decrease in IGF-1R activation. As shown in figure 3.6, exposure to 2-DG actually reduced IGF-1R beta chain tyrosine 1135/1136 phosphorylation in both basal and IGF-1 (130 nM) stimulated contexts and across all three cell lines. We also observed that AKT activation is not universally induced by 2-DG. Figure 3.6 shows that phospho-serine 473 AKT is increased in T47D cells and decreased in MCF-7 cells in basal 10% FBS conditions. In IGF-1 (130 nM) stimulated conditions, 2-DG does not appear to have a considerable effect on AKT activation in either MCF-7 or T47D cells. In HeLa cells, AKT phosphorylation is basally high and is not substantially altered in the presence of IGF-1 or 2-DG. These results indicate that 2-DG induced AKT activation is not universal and not IGF-1R dependent, as implied by Zhong et al. Moreover, 2-DG induced activation of 5' adenosine monophosphate activated protein kinase (AMPK) was consistently high across all cell lines (data not shown), which is in agreement with previously published results by Zhong et al. suggesting that 2-DG induced changes in AKT activation are AMPK independent (21).

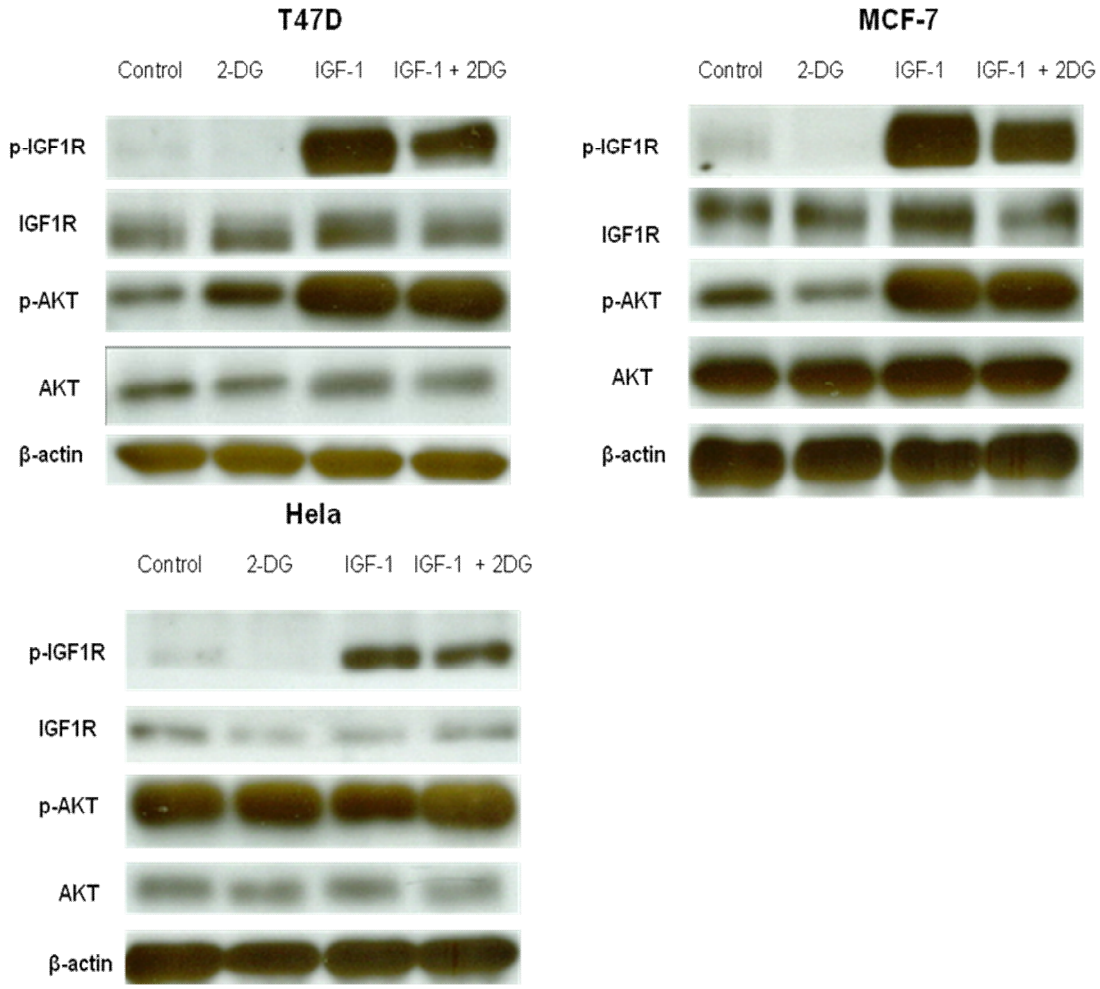


Figure 3.6: Western Blot assay of IGF-1R signaling. MCF-7, T47D and Hela cell lines were plated at 10^6 cells/well in 6-well plates in DMEM media containing 10% FBS. The following day, cells were treated with 10% FBS DMEM media containing 2-DG (25 mM), IGF-1 (130 nM) or both. Four hours later, cells were harvested and levels of signaling proteins were assayed by Western Blot.

3.5 Discussion

Our main objective was to investigate the effects of 2-DG and glucose on IGF-1:IGFBP-3 binding, as a follow-up to a recent publication by Zhong and colleagues (10). They showed that a concentration of 25 mM 2-DG was sufficient to disrupt more than 60% of IGF-1:IGFBP-3 complexes, a finding which, if confirmed, would have considerable impact both on IGF and cancer research. The reported finding that 2-DG exposure is associated with higher free IGF-1 concentrations challenges its utility as an antineoplastic agent and even its utility as an experimental strategy to selectively inhibit glycolysis without affecting other aspects of cellular physiology. It became important to determine if its relatively well characterized inhibition of glycolysis would predominate over this newly described stimulation in IGF signaling.

We sought to extend the finding of by Zhong and colleagues (10), by indentifying changes in affinity constants and by evaluating the potential effects of glucose, obviously structurally related to 2-DG, on IGF-1:IGFBP-3 complex formation. Using ELISA, we have shown that IGF-1:IGFBP-3 binding is unaltered by either 2-DG or glucose at various concentrations both within and exceeding physiologic range. The assay was performed both in different orientations and in different buffer systems with identical conclusions.

To date, several groups have used SPR to examine the interaction between IGF-1 and its binding proteins (19,20). Similar to the assay design detailed in previous reports, our SPR results are based upon IGF-1 injected over immobilized IGFBP-3 at a high flow rate (75 µl/min) and low signal range (<150 RU) to minimize any potential mass transport effects. Global fitting of the titration series to a 1:1 kinetic model with or without mass transport effects yielded similar outcomes. Our kinetic estimates were biologically-relevant (e.g. k_a ~10^3 – 10^7 $M^{-1}s^{-1}$ and k_d ~10^{-1} – 10^{-6} s-1 for typical protein interactions) and correlated well with values previously reported (19, 20) (i.e. overall affinity between IGF-1 and IGFBP-3 is sub-nanomolar). Ultimately, the presence of excess glucose or 2-DG

failed to significantly alter the binding interaction between IGF-1 and immobilized IGFBP-3 in our SPR assay.

Using Mass Spectrometry, we demonstrated for the first time that a newer "intensity fading" approach (17) is appropriate for the study of IGF-IGFBP complexes. While the resultant signal for the complexes did not match the anticipated theoretical predictions, this issue has been encountered by others (16,17) and may be the result of higher ionization energies required for complexes compared to the individual protein and its binding partner alone. Nevertheless, our intensity-fading experiments clearly show that the signal corresponding to IGF-1 was decreased in a dose-dependent manner upon IGFBP-3 addition and that 2-DG or glucose had no effect compared to the control MS spectra.

Upon examination of 2-DG induced changes in IGF-1R signaling in MCF-7, T47D and HeLa cell lines, we were unable to confirm the previously published finding that exposure to 2-DG induces IGF-1R activation(10). In fact, we observed that 2-DG treatment reduces phosphorylation of IGF-1R in all three cell lines tested. This finding provides further evidence against the hypothesis that 2-DG disrupts IGF-1:IGFBP-3 complex formation which leads to activation of the IGF-1R. Moreover, the fact that phospho-serine 473 AKT levels changed in a cell specific manner and were uncorrelated with phospho-IGF-1R levels supports the hypothesis that 2-DG induced changes in AKT activation are independent of IGF-1R in these cell lines.

In conclusion, we have utilized three unique experimental strategies to demonstrate that 2-DG does not alter the binding interaction between IGF-1 and IGFBP-3. While our direct measures of IGF-1:IGFBP-3 complex formation contrast with the experimental findings reported by Zhong et al., we suspect that the commercial assay (which was designed to measure free IGF-1 in serum samples) utilized in their study may have been limited by its ability to provide only an indirect measure of IGF-1:IGFBP-3 complex formation. The mechanism responsible for 2-DG induced increase in IGF-1R signaling observed by Zhong

and colleagues (10, 21) in some cell lines remains unknown. However, our results using several independent methods do not support the hypothesis of a universal mechanism involving IGF-1:IGFBP-3 complex disruption, but rather suggest a mechanism involving cell specific intracellular signaling differences.

Chapter References

1. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. Nat Rev Cancer 2008;8:915-28.

2. Laviola L, Natalicchio A, Giorgino F. The IGF-I signaling pathway. Curr Pharm Des 2007;13:663-9.

3. Collett-Solberg PF, Cohen P. Genetics, chemistry, and function of the IGF/IGFBP system. Endocrine 2000;12:121-36.

4. Vincent AM, Feldman EL. Control of cell survival by IGF signaling pathways. Growth Horm IGF Res 2002;12:193-7.

5. LeRoith D, Roberts CT, Jr. The insulin-like growth factor system and cancer. Cancer Lett 2003;195:127-37.

6. Ghosh P, Dahms NM, Kornfeld S. Mannose 6-phosphate receptors: new twists in the tale. Nat Rev Mol Cell Biol 2003;4:202-12.

7. Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev 2002;23:824-54.

8. Hwa V, Oh Y, Rosenfeld RG. The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocr Rev 1999;20 :761-87.

9. Jones JI, Clemmons DR. Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev 1995;16:3-34.

10. Zhong D, Xiong L, Liu T, Liu X, Liu X, Chen J, et al. The glycolytic inhibitor 2-deoxyglucose activates multiple prosurvival pathways through IGF1R. J Biol Chem 2009;284:23225-33.

11. WICK AN, DRURY DR, MORITA TN. 2-Deoxyglucose; a metabolic block for glucose. Proc Soc Exp Biol Med 1955;89:579-82.

12. WICK AN, DRURY DR, NAKADA HI, WOLFE JB. Localization of the primary metabolic block produced by 2-deoxyglucose. J Biol Chem 1957;224:963-9.

13. Chen W, Gueron M. The inhibition of bovine heart hexokinase by 2-deoxy-D-glucose-6-phosphate: characterization by 31P NMR and metabolic implications. Biochimie 1992;74:867-73.

14. Singh D, Banerji AK, Dwarakanath BS, Tripathi RP, Gupta JP, Mathew TL, et al. Optimizing cancer radiotherapy with 2-deoxy-d-glucose dose escalation studies in patients with glioblastoma multiforme. Strahlenther Onkol 2005;181:507-14.

15. Myszka DG. Improving biosensor analysis. J Mol Recognit 1999;12:279-84.

16. Levitt RJ, Georgescu MM, Pollak M. PTEN-induction in U251 glioma cells decreases the expression of insulin-like growth factor binding protein-2. Biochem Biophys Res Commun 2005;336:1056-61.

17. Bolbach G. Matrix-assisted laser desorption/ionization analysis of non-covalent complexes: fundamentals and applications. Curr Pharm Des 2005;11:2535-57.

18. Yanes O, Nazabal A, Wenzel R, Zenobi R, Aviles FX. Detection of noncovalent complexes in biological samples by intensity fading and high-mass detection MALDI-TOF mass spectrometry. J Proteome Res 2006;5:2711-9.

19. Yanes O, Villanueva J, Querol E, Aviles FX. Detection of non-covalent protein interactions by 'intensity fading' MALDI-TOF mass spectrometry: applications to proteases and protease inhibitors. Nat Protoc 2007;2:119-30.

20. Zhong D, Liu X, Schafer-Hales K, Marcus AI, Khuri FR, Sun SY, et al. 2-Deoxyglucose induces Akt phosphorylation via a mechanism independent of LKB1/AMP-activated protein kinase signaling activation or glycolysis inhibition. Mol Cancer Ther 2008;7:809-17.

21. Beattie J, Phillips K, Shand JH, Szymanowska M, Flint DJ, Allan GJ. Molecular interactions in the insulin-like growth factor (IGF) axis: a surface plasmon resonance (SPR) based biosensor study. Mol Cell Biochem 2008;307:221-36.

22. Vorwerk P, Hohmann B, Oh Y, Rosenfeld RG, Shymko RM. Binding properties of insulin-like growth factor binding protein-3 (IGFBP-3), IGFBP-3 N- and C-terminal fragments, and structurally related proteins mac25 and connective tissue growth factor measured using a biosensor. Endocrinol 2002;143:1677-85.

Chapter 4

Effects of BI836845, a candidate therapeutic antibody against IGF-1 and IGF-2, on IGF-1:IGFBP-3 complexes

Matei Mireuta[1], Paul J. Adam[2], Elena Birman[1], Melinda Barmash[1] and Michael Pollak[1]

[1] Departments of Medicine and Oncology, Lady Davis Institute for Medical Research and Segal Cancer Center, Jewish General Hospital and McGill University, Montreal, Quebec, Canada H3T 1E2

[2] Boehringer Ingelheim RCV GmbH & Co KG, Dr. Boehringer Gasse 5-11, A-1121, Vienna, Austria

This manuscript has been submitted to Boehringer Ingelheim for internal review as per our material transfer agreement, and upon approval will be submitted for publication.

Preface

For the last part of the thesis, we studied aspects of IGFBP physiology relevant to a novel drug candidate for cancer treatment. A high affinity antibody (BI836845) cross-specific to IGF-1 and IGF-2 has been developed by Boehringer Ingelheim as a potential therapeutic agent in the treatment of cancer. As discussed in the literature review, IGFs have roles in carcinogenesis and progression of neoplasia and the IGF axis has therefore been considered as a potential target in the context of cancer therapy. Agents that interfere with IGF signaling have been developed over the years and fall into two categories: IGF-1R specific antibodies which neutralize the receptor or small tyrosine kinase inhibitors which prevent receptor activation. The approach by which ligands are targeted with neutralizing antibodies is relatively novel and may influence IGFBP physiology. We hypothesized that in the presence of high affinity anti-ligand antibodies, IGFs will shift from IGFBP complexes to antibody complexes and a large pool of free IGFBPs would be created. As discussed in chapter 1, several studies raised the possibility that IGFBPs may have biologic effects that are either IGF dependent or independent. We therefore studied the effect of BI836845 on IGFBP-3 using a mouse model as an initial step towards investigation of physiologic effects of this antibody in humans.

4.1 Abstract

Insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) are potent mitogens acting through the IGF-1 receptor (IGF-1R). The importance of the IGF system in neoplasia has been demonstrated in several models and IGF-1 signaling has become a target for drug development. The drug candidate BI836845 is a fully human IgG1 ligand neutralizing antibody that cross reacts with IGF-1 and IGF-2. It has been shown to reduce both IGF-1R phosphorylation and cellular proliferation in preclinical studies and now is being evaluated in clinical trials. In rodent studies, administration of BI836845 leads to large increases in total IGF-1 concentration in serum. In the light of this observation, it is important to quantify the level of IGF-1:BI836845 complexes in serum in relation to IGF-1 bioactivity. In this report, we develop a novel technique to measure ligand: BI836845 binding and we apply it to a mouse model in various contexts. We show that although large increases in total serum IGF-1 levels are observed, the vast majority of ligand is present as a complex with BI836845. We show that, although the therapeutic antibody leads to an increase in free IGFBP-3 ex vivo due to ligand displacement, an increase in free IGFBP-3 is not observed in vivo. Finally, we show that BI836845 treatment induces an increase in growth hormone levels, a finding consistent with a pituitary compensation mechanism similar to that reported following treatment with IGF-1R specific antibodies.

4.2 Introduction

Insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) are peptide hormones similar in structure to insulin that regulate a variety of cellular activities, including metabolism, proliferation, and growth. The IGF-1 receptor (IGF-1R) binds both IGFs and initiates a signaling cascade that results in the activation of the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin pathway (PI3K/AKT/mTOR) (1-5). The IGF-2 receptor, which binds to IGF-2, does not possess intrinsic tyrosine kinase activity and is therefore considered as a sink that decreases IGF-2 bioactivity (6).

IGF actions are tightly modulated by a family of proteins called insulin-like growth factor-binding proteins (IGFBPs), of which IGFBP-1–6 have been characterized. The liver is the main source of serum IGFs and IGFBPs, but their expression is essentially ubiquitous. IGFBP-3 is the most abundant IGFBP in the circulation. It forms a ternary complex with IGFs and a third protein termed acid-labile subunit (7,8). Insulin-like growth factor-binding proteins are known to modulate actions of IGFs both in vitro and in vivo (7).

Activation of the IGF-1R is generally accepted to play an important role in carcinogenesis and progression of neoplasia through increases in cell growth and proliferation and inhibition of apoptosis (9). Therefore, the IGF axis represents a potential target for cancer therapy (10-12). Early candidate drugs that have been clinically evaluated for the treatment of neoplasia are IGF-1R specific antibodies (13). Despite encouraging early clinical data, definitive phase III clinical studies have been disappointing (14). IGF-1R tyrosine kinase inhibitors (13) and a newer class of ligand specific antibodies (15,16) have recently entered clinical trials.

The drug candidate BI836845 (Boehringer Ingelheim) (15) is a monoclonal, fully human dual specific IgG1 antibody with high affinity for both human and rodent

IGF-1 and IGF-2. It was shown in preclinical studies to inhibit phosphorylation of the IGF-1R in vitro and to lead to decreased proliferation of a panel of cancer cell lines. In rodents, animal weight was reduced in a dose-dependent manner after a 13 week period of weekly intra-peritoneal administration of BI836845. Additionally, experimental tumor volumes were consistently lower in mice treated with BI836845 as compared to vehicle. Interestingly, BI836845 treatment also lead to substantial increases of total IGF-1 serum levels in both normal and immunocompromised rodents (15). This effect may be the result of a host compensatory mechanism by which growth hormone levels rise as a consequence of the relaxation of the inhibitory IGF-1 feedback-loop to the pituitary. This effect is common with IGF-1R specific antibodies and was recently reviewed (13). On the other hand, it is also plausible that the half-life of IGF-1 is extended by binding to BI836845 and increased serum levels may represent a decrease in IGF-1 degradation/elimination. Of course, these mechanisms are not mutually exclusive.

In the present study, we further characterize the therapeutic antibody BI836845 by examining its effects in a mouse model. We develop a novel technique to measure IGF-1:BI836845 complexes in serum and we show that BI836845 has the ability to sequester the vast majority of IGF-1 suggesting that presence of additional IGF-1 binding sites in serum are the major cause for increases in total IGF-1 levels.

4.3 Materials and Methods

Materials

BI836845 was kindly provided by Dr. Paul Adam (Boehringer Ingelheim, Vienna, Austria). Recombinant mouse IGF-1 and IGFBP-3 were purchased from R&D Systems (Minneapolis MN, USA). Control non specific human IgG1 isotype antibody was purchased from Cell Sciences (Canton, MA, USA) and C57 BL/6 mouse pooled serum was purchased from GeneTex (Irvine, CA, USA).

Animals

C57 BL/6 mice 6-8 weeks of age were purchased from Charles River Laboratories (St-Constant, Quebec, Canada) and were randomized into 4 groups for each experiment. In the first study, 3 mice received 10 mg/kg BI836845, 3 mice received 30 mg/kg BI836845, 3 mice received 100 mg/kg BI836845 and 4 mice received vehicle. The treatments were performed intra-peritoneally alternatively every 3^{rd} or 4^{th} day, for 4 doses and animals were sacrificed 4 days after the last dose. A sample of plasma was obtained before sacrifice and total mouse serum was collected by cardiac puncture and further used in column processing experiments. In the second study, 12 mice were treated intra-peritoneally with 100mg/kg BI836845 and 3 mice were sacrificed after 2 hours, 3 mice were sacrificed after 6 hours, 3 mice were sacrificed after 24 hours and 3 mice were sacrificed after 48 hours. 4 control mice received vehicle and 2 mice were sacrificed after 48 hours. Again, a plasma sample was obtained before sacrifice and total serum was collected by cardiac puncture and used in subsequent column processing experiments. Plasma samples were used of IGFBP-3 ELISA experiments.

Protein A agarose columns

Small 0.2ml Nab Protein A-agarose plus columns and Protein A binding buffer were purchased from Thermo Fischer (Ottawa, Canada). Since the columns are prepacked with more resin than is optimal, they were emptied while the resin was

stored separately. For each condition, a column was packed with 140 µl of resin, centrifuged at 5000 RPM for 1 minute to remove storage solution and then washed three times with Protein A binding buffer. After the last centrifugation, 150 µl of serum were added to the resin and mixed with thorough up and down movements for 10 minutes. The columns were then centrifuged and the first fraction was collected and termed flow-through. A volume of 400 µl of elution buffer (100 mM glycine at ph 2.2) was applied to the resin for dissociating antibody:bead complexes. After a brief mix, the columns were centrifuged and the second fraction termed elution 1 was collected. The process was repeated for elution fractions 2 and 3.

ELISA assays and variants

IGF-1 and IGFBP-3 immunoassay kits were purchased from R&D systems and a human IgG1 immunoassay kit was purchased from Abcam (Cambridge, MA, USA) and were used according to manufacturers specifications except for the following experiments.

For the IGF-1 assay, the high temperature pretreatments were achieved by heating a sample of the first required serum dilution and using this heated sample to generate the second dilution.

For immunoprecipitation, undiluted serum samples were incubated in microwell plates precoated with IGFBP-3 specific antibody and incubated for two hours. Then, wells were washed and the immunoprecipitated material was dissociated with a solution containing 100 mM glycine at ph 2.3. The samples were neutralized and used in IGF-1 or IGFBP-3 immunoassays.

Western Blotting

Immunoblotting experiments were performed as previously described (Chapter 2) and IGF-1 specific primary antibody was purchased from R&D systems and IGFBP-3 specific antibody was obtained from Santa Cruz (Santa Cruz, CA, USA).

4.4 Results

Use of a protein A column to remove BI836845 from mouse serum

In order to study the distribution of IGF-1 between complexes with the therapeutic IgG1 class antibody BI836845 and serum IGFBPs, we conducted an immunodepletion experiment using protein A agarose conjugates and C57 BL/6 mouse serum to which different amounts of BI836845 or a non-specific control human IgG1 were added. The immunodepletion method is described in figure 1 and requires running serum through a column containing protein A-agarose, collecting the flow-through and then eluting three fractions under suitable conditions. Although protein G has a higher affinity for human IgG1 than protein A (17), we chose the latter because it has a lower affinity for endogenous mouse IgGs (18).

Various amounts of BI836845 or of control IgG1 antibody were added to mouse serum samples to reach a final concentration of 0 to 300 µg/ml BI836845 or of 300 µg/ml control antibody. Samples were then incubated 16 hours at 37 ºC and then subjected to the column treatment depicted in figure 4.1. Each condition yielded four fractions labeled flow through, elution 1, elution 2 and elution 3.

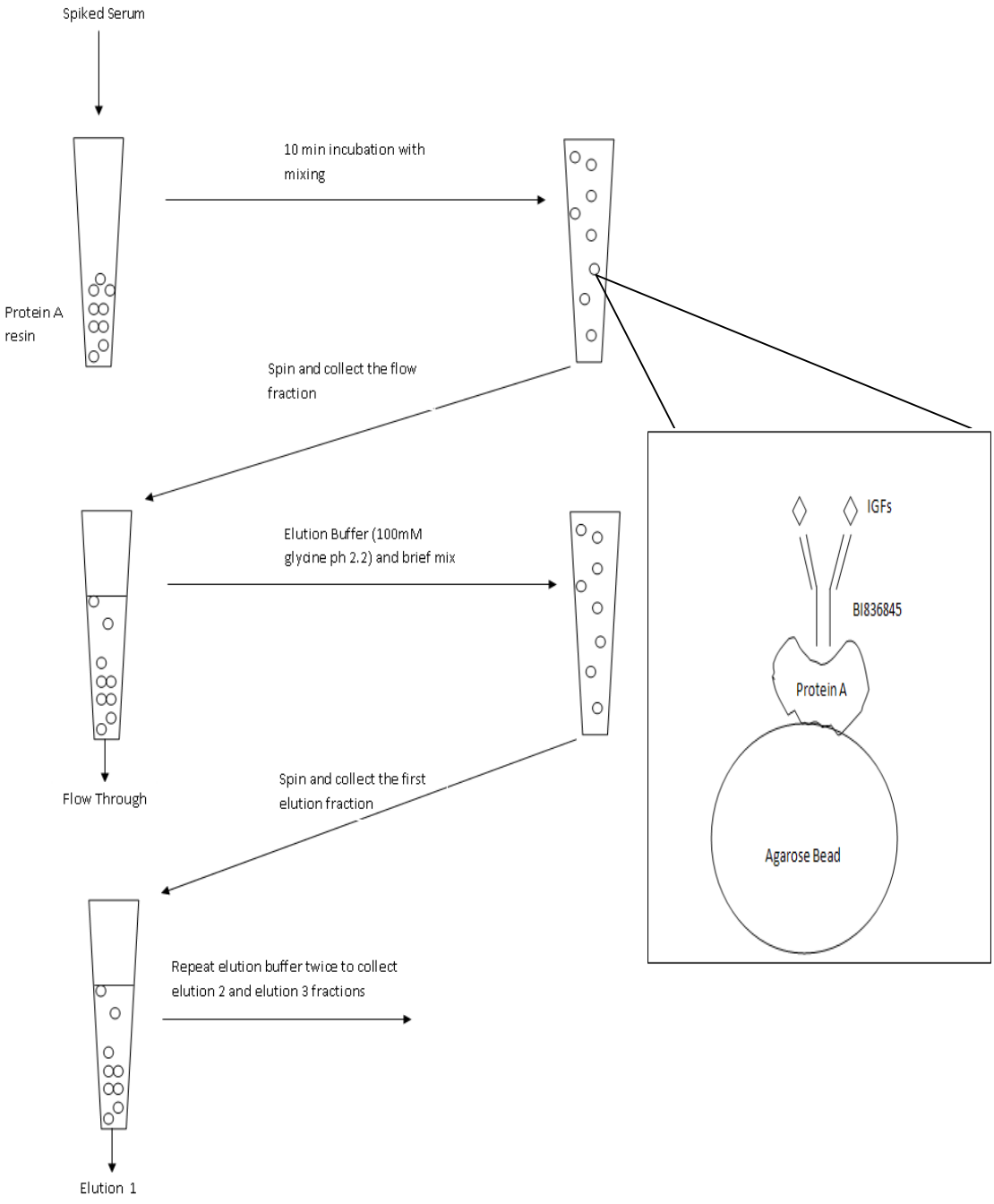
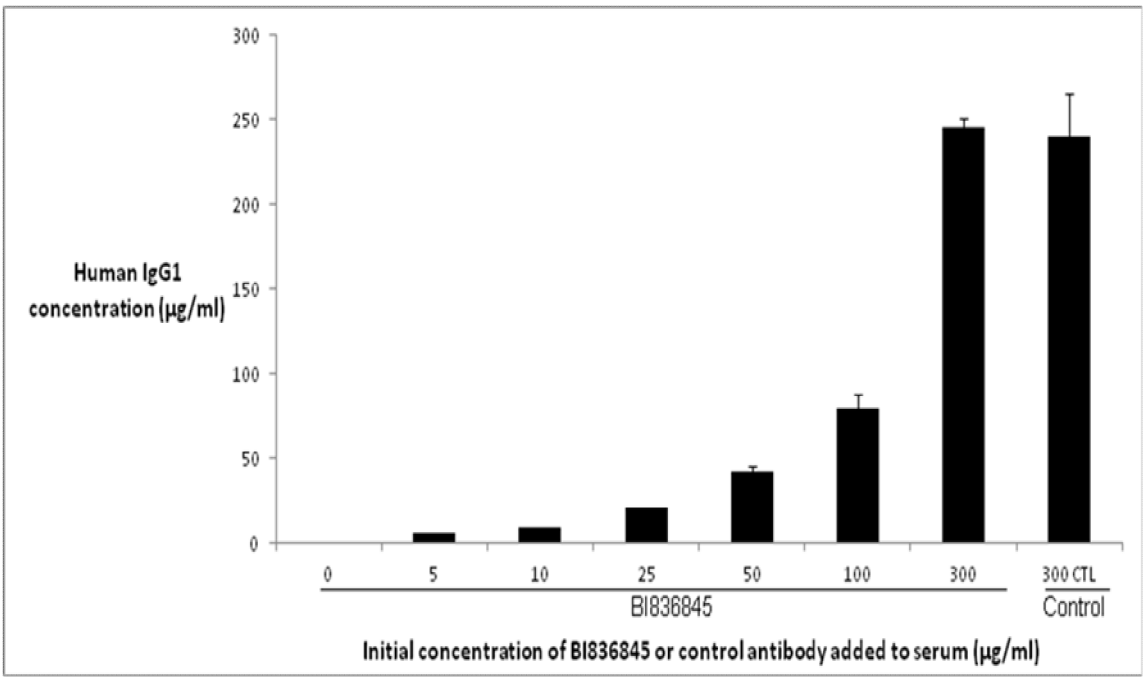


Figure 4.1: Schematic representation of the technique described in the material and methods section.

Then, human IgG1 concentration was measured in each fraction as well as in the total sample prior to column loading. Figure 4.2A shows that IgG1 levels measured in the mouse serum after the 16 hour incubation were comparable to the initial concentration added, indicating that the human IgG1 degradation was minimal. More importantly, figure 4.2B indicates that the experimental method was suitable for the effective removal of BI836845 from serum as less than 5% of the added antibody was found in the flow through. Also shown is the amount of IgG1 present in the elution fractions, with most BI836845 eluting in the first fraction and a much smaller amount eluting in the second fraction. The third elution values are not shown as they were consistently 0 across all conditions. The recovery rate was calculated as the sum of the three fractions (Flow through + Elution 1 + Elution 2) divided by the amount in total sample prior to column loading and yielded values between 83% and 92% for all conditions. Throughout the results section, the concentration of analytes in elution fractions (400 µl) has been normalized to the volume of serum added to the columns (150 µl) to allow for direct comparison (i.e. multiplication by a factor of 2.66).

A)



B)

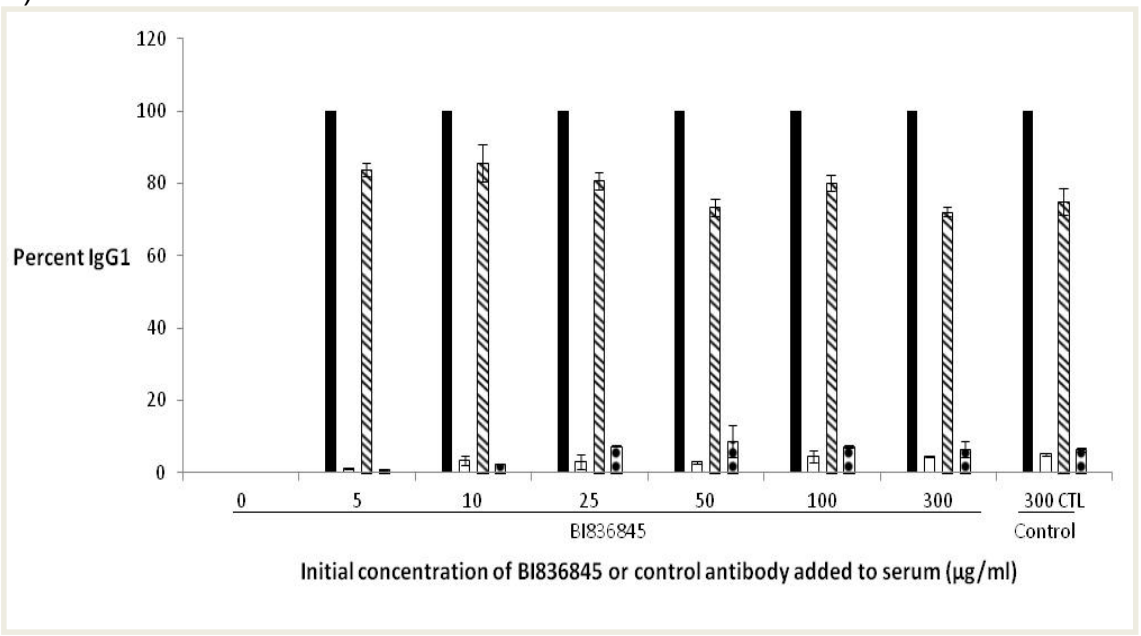


Figure 4.2: Concentration of human IgG1 in column fractions as measured by ELISA. A) Human IgG1 concentration measured by ELISA in mouse serum incubated for 16 hours at 37°C with respect to initial antibody concentrations added. B) The percentage of IgG1 measured by ELISA in flow through (white), first elution (striped) and second elution (dotted) fractions with respect to total fraction (black). Error bars indicate standard deviation of measurements from at least two independent column processing experiments.

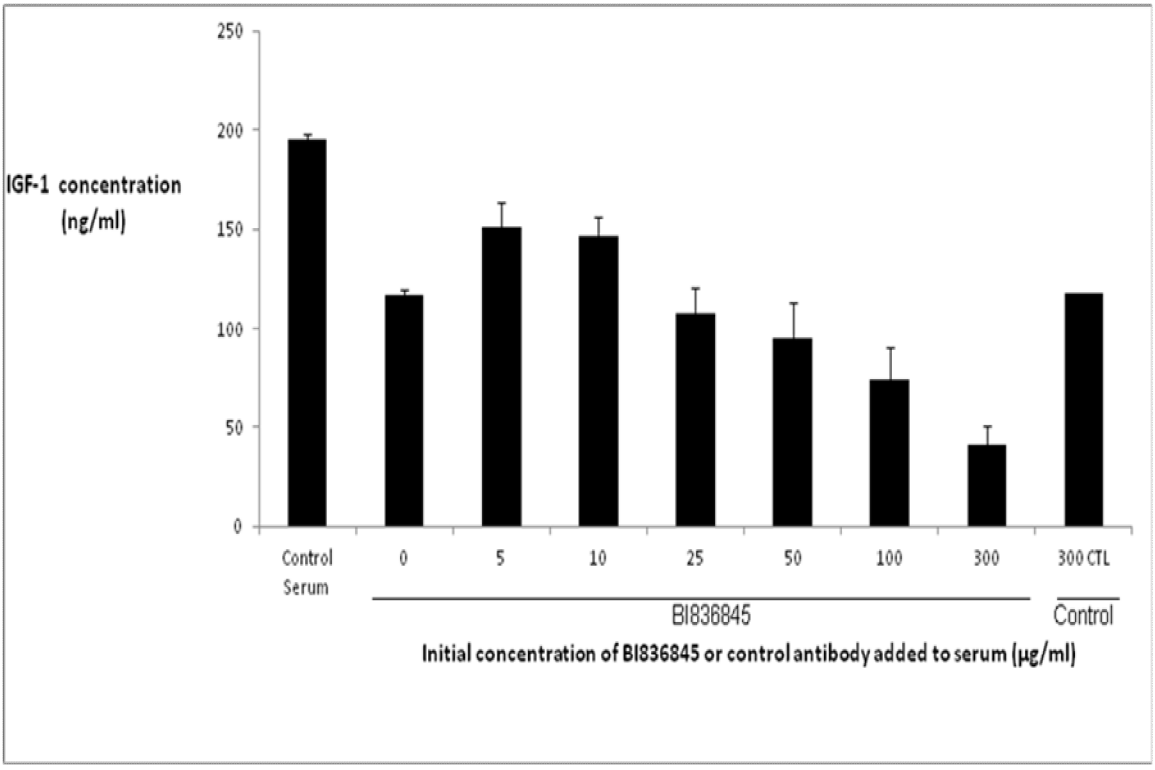
Minimization of interference of IGF-1 ELISA by BI836845 using preincubation at 80.6 ºC

We then used an ELISA assay to measure IGF-1 in serum samples to which BI836845 was added and observed that the therapeutic antibody interfered with the IGF-1 detection system (Figure 4.3A). This artifact is anticipated given the fact that the technique used to measure IGF-1 is a sandwich ELISA and that BI836845 is a high affinity antibody for the analyte. As this interference is anticipated to occur with most immunoassays, we developed a sample pretreatment technique that would dissociate the antibody/ligand complexes, based on the relative thermal stability of IGF-1. We had evidence, both from the literature (19) and pilot experiments, that IGF-1 is heat stable. On the other hand, IgGs are relatively heat labile and, at least for rat IgG1, the denaturing temperature was reported to be 60 ºC for the F_{ab} domain and 71 ºC for the F_c domain (20). We therefore incubated serum samples containing 0 µg/ml BI836845 or 300 µg/ml BI836845 at increasing temperatures for 15 minutes in order to denature BI836845, with the expectation that most IGF-1 would survive this treatment.

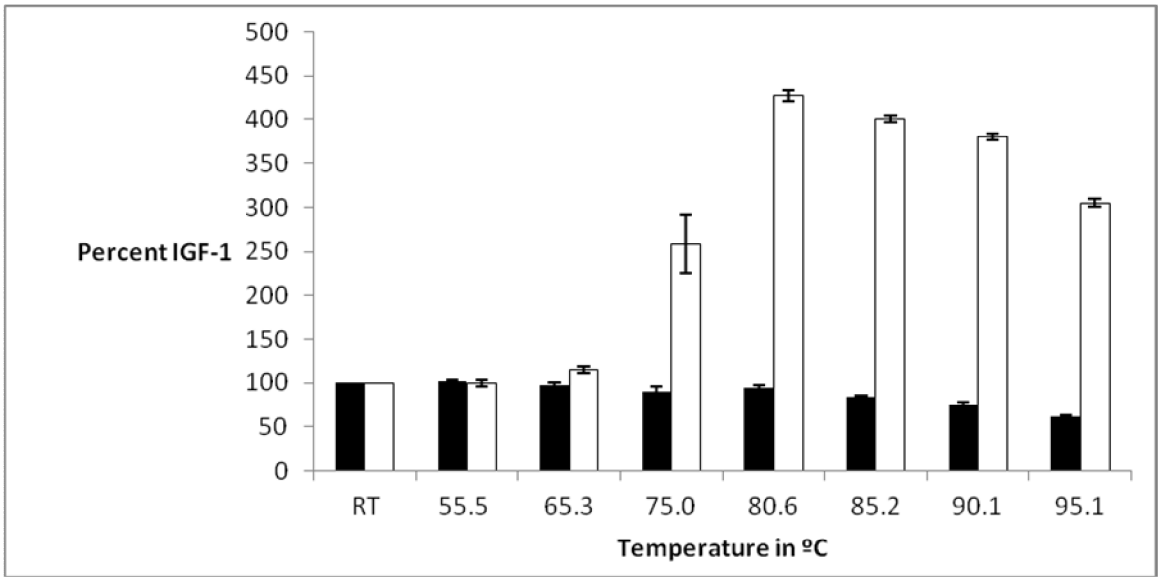
Figure 4.3B shows that IGF-1 in the absence of BI836845 is affected by heat to a certain extent, denaturing in a temperature dependent fashion and losing about 37% immunoreactivity at the highest temperature tested (i.e. 95 ºC). However, incubation at increasing temperatures of the serum sample containing 300 µg/ml BI836845 reveals more IGF-1 immunoreactivity as the IgG1 is denatured with increasing temperature. At temperatures greater than 80.6 ºC, the revealed IGF-1 immunoreactivity degrades similarly to the control sample. Thus, incubation at around 80 ºC reveals almost all of the previously obscured IGF-1 immunoreactivity. Also, at this temperature very little (<10%) actual IGF-1 immunoreactivity is lost to denaturing as evidenced in the 0 µg/ml BI836845 sample. BI836845 is likely irreversibly denatured after 15 minutes incubation at 80.6 ºC, because no signal is observed for samples pretreated in this fashion when using the same IgG1 ELISA assay as in figure 2 (data not shown).

Figure 4.3C shows the variation in absolute amount of IGF-1 with and without the pre incubation at 80.6 ºC. An additional feature that is revealed by the pretreatment is that BI836845 has an effect on IGF-1 degradation in serum ex vivo. The sample denoted “Control serum” refers to serum that was not manipulated in any way, while the 0 µg/ml BI836845 sample was incubated for 16 hours at 37 ºC. In the control serum sample, the initial concentration of IGF-1 is ~200 ng/ml. This drops to ~100 ng/ml in the 0 µg/ml BI836845 sample, in contrast to samples incubated in the presence of BI836845 in which ~150ng/ml IGF-1 is detected. This demonstrates that BI836845 inhibits IGF-1 degradation in serum ex vivo.

A)



B)



C)

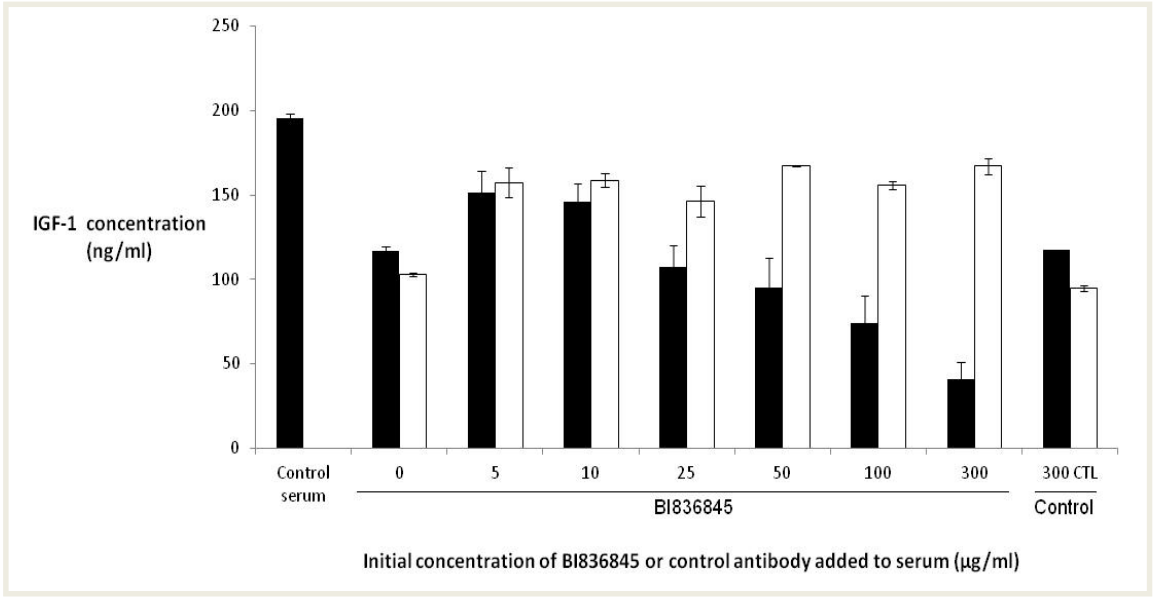
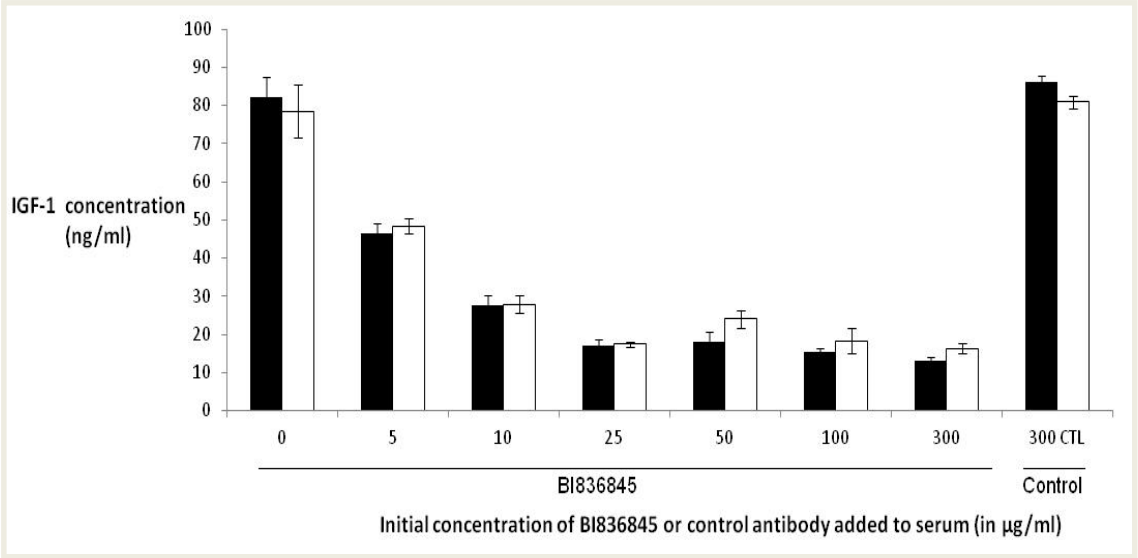


Figure 4.3: Effect of a temperature pretreatment on IGF-1 detection by ELISA. A) Mouse IGF-1 concentration measured by ELISA in mouse serum incubated for 16 hours at 37 °C relative to initial BI836845 or control antibody concentrations added. B) The percentage of mouse IGF-1 immunoreactivity detected as a function of sample temperature pretreatment relative to room temperature (RT) in mouse serum samples containing either no BI836845 (black) or 300 µg/ml BI836845 (white). C) Mouse IGF-1 concentrations measured by ELISA with no pretreatment (black) or following an 80.6°C pretreatment (white) as a function of initial BI836845 or control antibody concentration. Error bars indicate standard deviation of measurements from at least two independent incubation experiments.

IGF-1 is associated with BI836845 in serum

At this stage, we concluded that we could measure IGF-1 reliably in the presence of BI836845 using the 80.6 ºC incubation pretreatment prior to the ELISA. Figure 4.4A shows the amount of IGF-1 present in the flow-through fraction of serum passed through the protein A column as we varied BI836845 concentration, measured with and without the 80.6 ºC incubation. As expected, the amount of IGF-1 retained in the column increases as initial concentrations of BI836845 added to the serum are raised. Figure 4.4B indicates that the vast majority of bound IGF-1 elutes in the first elution fraction, which is also the fraction containing the vast majority of bound BI836845 (Figure 4.2B). The third elution fraction is not shown as it was consistently 0 across all conditions. The recovery rate of IGF-1, measured again as the sum of three fractions (Flow through + Elution 1+ Elution2) divided by the amount prior to column loading, varied between 77% and 97%, with an average of 84%.

A)



B)

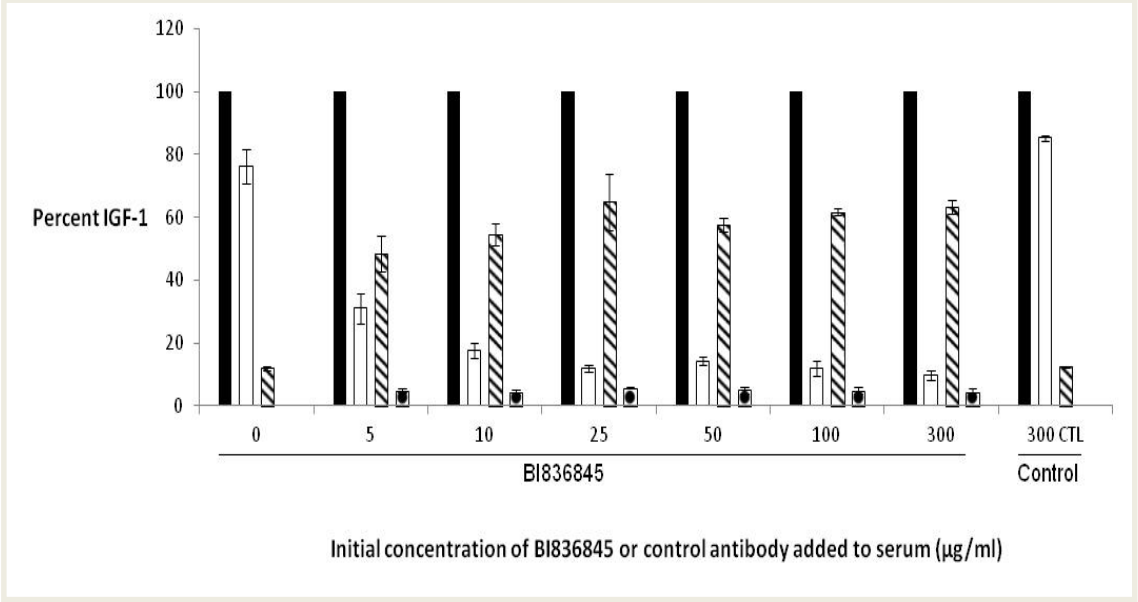


Figure 4.4: Concentration of mouse IGF-1 in column fractions as measured by ELISA. A) Mouse IGF-1 concentration in the flow-through fraction detected by ELISA with no pretreatment (black) or following an 80.6°C pretreatment (white) according to initial BI836845 or control antibody concentration. B) The percentage of IGF-1 measured by ELISA (with 80.6°C pretreatment) in flow through (white), first elution (striped) and second elution (dotted) fractions with respect to total fraction (black) after column processing. Error bars indicate standard deviation of measurements from at least two independent column processing experiments.

The data shown in figure 4.4 suggest a loss of IGF-1 after column filtration as evidenced by the fact that about 75-80% of initial IGF-1 is present in the flow-through of samples containing no BI836845 or 300 µg/ml of control human IgG1. This is not the outcome of non-specific binding because prior blocking of the columns with either bovine serum albumin or Tween-20 had no effect on this measurement (data not shown). On the other hand, the results reported in figure 4.4 are based on the assumption that the column volume is negligible. In reality, the measured amount of fluid inherently retained in a column (after thorough centrifugation) is around 50 µl, and this volume acts as a diluent, particularly in the initial step of the technique. Table 4.1 illustrates a correction process for this dilution event and indicates that this procedure fully accounts for the apparent loss of IGF-1 observed for samples containing no BI836845 or 300 µg/ml of control human IgG1. Therefore non-specific binding of ligand to the column is not a major concern. Furthermore, this adjustment, although warranted from experimental observations, has a minor impact on the estimated amount of IGF-1/BI836845 complexes for the remaining conditions of table 4.1.

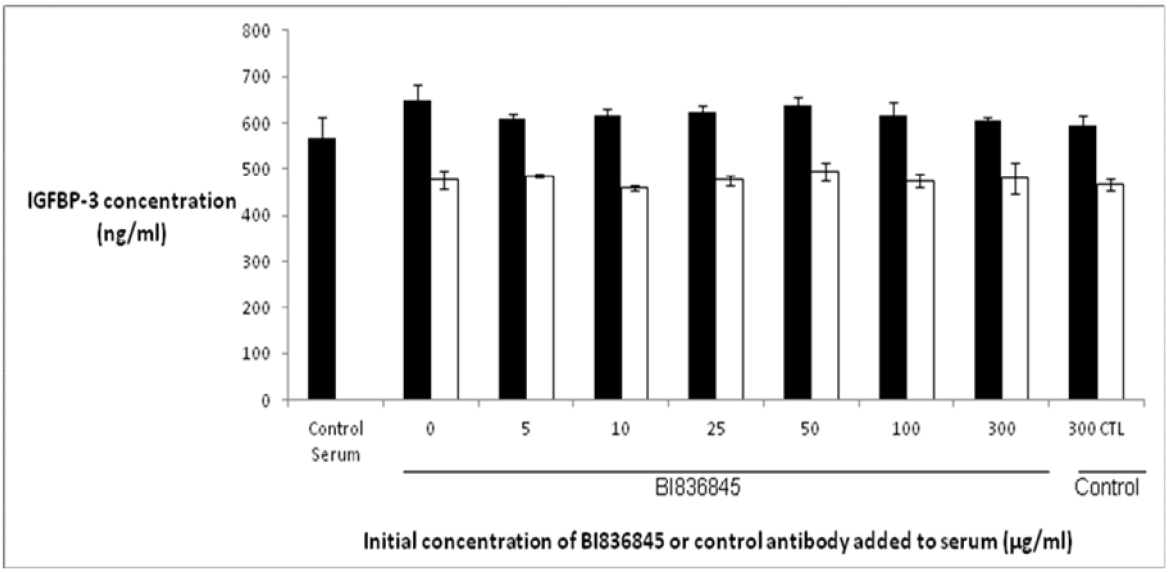
Condition	Initial concentration of IGF- 1 (ng/ml)	Initial concentration of IGF-1 (ng/ml) corrected for column volume by a factor of 0.75 (150 µl of serum in 200 µl final volume)	Concentration of IGF-1 in flow-through (ng/ml)	Percent IGF-1 in flow-through corrected for column volume
0 ug/ml BI836845	103.02	77.27	78.51	101.5
5 ug/ml BI836845	157.43	118.07	48.39	41.3
10 ug/ml BI836845	158.76	119.07	27.82	23.4
25 ug/ml BI836845	146.08	109.56	17.43	16
50 ug/ml BI836845	167.53	125.65	23.99	19.1
100 ug/ml BI836845	155.63	116.72	18.33	15.7
300 ug/ml BI836845	167.11	125.33	16.31	13.1
300 ug/ml CTL IgG1	94.92	71.19	80.92	113.7

Table 4.1: A correction process for flow-through measurements taking into account initial column volume.

Effect of BI836845 on IGF-1/IGFBP-3 binding

In addition to the IGF-1 observations, a key consideration was the impact of BI836845 on IGFBP-3 distribution between the flow-through and elution fractions. Figure 4.5A shows that IGFBP-3 is not retained by the protein A agarose resin for any of the BI836845 concentrations tested (the minor decrease in concentration in the flow fraction is the result of the dilution event previously described). This finding suggests that ternary complexes of IGF-1:IGFBP-3:BI836845 are not formed and that IGF-1 shifts gradually from IGFBP-3 to BI836845 as the concentration of the therapeutic antibody is increased. Figure 4.5B is a confirmation of the validity of earlier IGF-1 and IGFBP-3 measurements by a Western Blot technique.

A)



B)

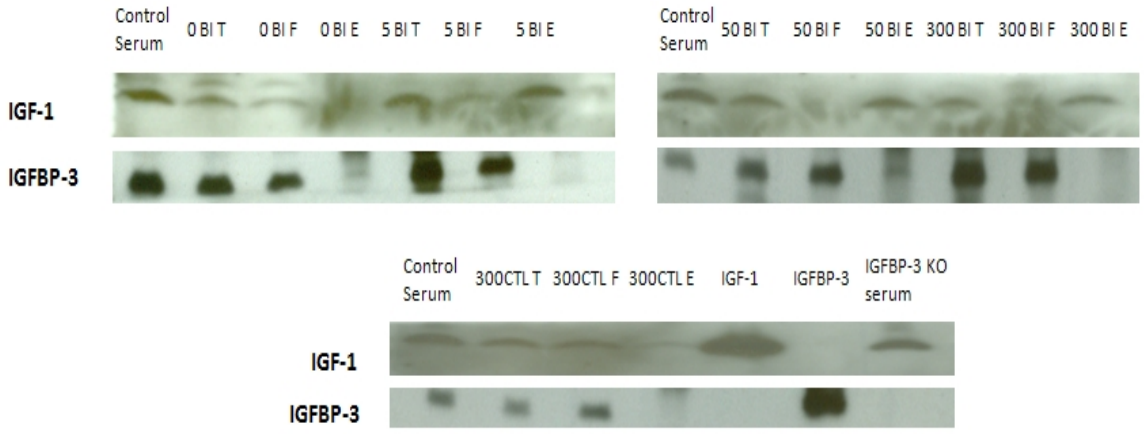


Figure 4.5: Concentration of mouse IGF-1 and IGFBP-3 in column fractions as measured by ELISA and WB.A) Mouse IGFBP-3 concentrations detected by ELISA in the total fraction (black) or the flow-through fraction (white) after column processing with respect to initial BI836845 or control antibody concentrations added. Error bars indicate standard deviation of measurements from at least two independent column processing experiments B) Western Blot estimate of IGF-1 and IGFBP-3 in the total (T), flow through (F) or elution 1 (E) fractions in 2ul volumes of samples containing 0 µg/ml BI836845 (0 BI), 5 µg/ml BI836845 (5 BI), 50 µg/ml BI836845 (50 BI), 300 µg/ml BI836845 (300 BI) or 300 µg/ml control IgG1 (300 CTL). The IGF-1 (25 ng of recombinant mouse IGF-1), IGFBP-3 (100ng of recombinant mouse IGFBP-3 and IGFBP-3 KO serum (2ul of serum from an IGFBP-3 knock out mouse) were loaded as controls. The results are representative of at least three immunoblots from at least two independent column processing experiments.

In order to confirm earlier results relative to IGFBP-3 distribution between column fractions, we isolated total IGFBP-3 from BI836845 treated mouse serum as described in figure 4.6 and then measured total associated IGF-1. Figure 4.6 shows that as the concentration of BI836845 increases, less IGF-1 is bound to IGFBP-3 indicating that IGFBP-3 exists in free form. Interestingly, the addition of 5 µg/ml antibody shows almost no effect on IGFBP-3 associated IGF-1 levels despite the fact that more than half of total IGF-1 is bound to BI836845 in this sample (Table 4.1). As the affinity of IGFBP-3 for IGF-1 is among the highest in the IGFBP family (21), we speculate that, in the presence of BI836845, IGF-1 may dissociate in a first step primarily from IGFBPs other than IGFBP-3.

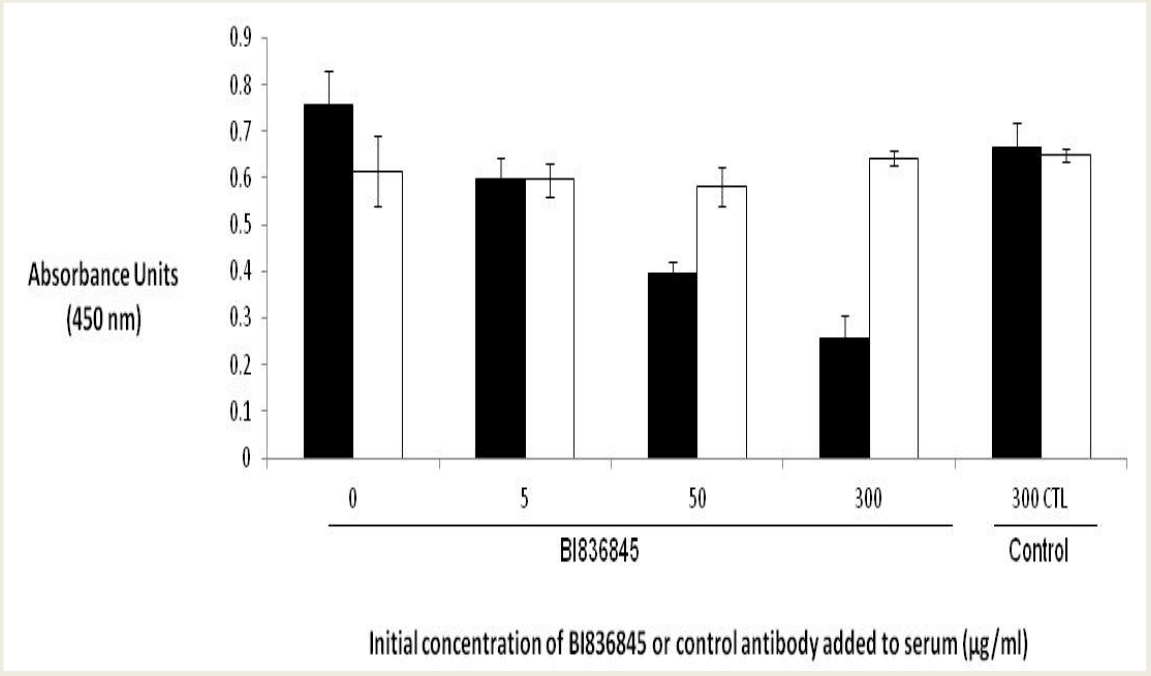


Figure 4.6: Mouse serum levels of free IGFBP-3. Samples of undiluted serum containing different concentrations of BI836845 or 300 µg/ml control IgG1 were incubated in microplate wells coated with IGFBP-3 specific antibody. Molecular species bound to the anti-IGFBP-3 coated wells were eluted in an acidic condition (100 mM glycine, ph 2.3), neutralized and amounts of IGF-1 (black) or IGFBP-3 (white) were quantified by ELISA. Error bars represent standard deviation of measurements from at least two independent experiments.

In order to further examine the effect of BI836845 on IGF-1 degradation observed in figure 3, we incubated mouse serum with different concentrations of the antibody for an additional 24 hours at 37 ºC. Figure 4.7 shows that the effect of BI836845 on IGF-1 degradation in serum ex vivo is substantial and dose-dependent at this later time point. The control non-specific IgG1 displayed no effect, as anticipated. This represents additional confirmation of IGF:BI836845 binding and is consistent with the data obtained from the column processing experiments which showed that the majority of IGF-1 is bound to the therapeutic antibody.

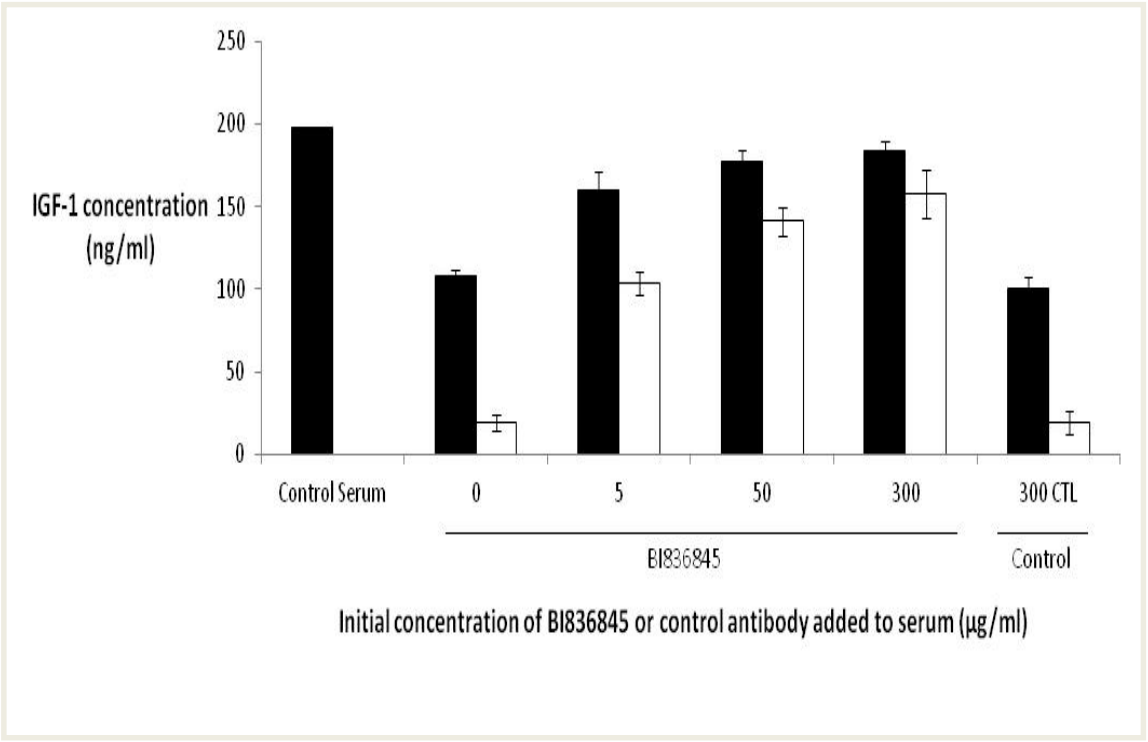


Figure 4.7: Mouse serum IGF-1 degradation. Measurements of IGF-1 concentration by ELISA in serum samples incubated for 16 hours at 37C (black) or 40 hours at 37 C (white) relative to initial BI836845 or control antibody concentrations added. Error bars represent the standard deviation of measurements from at least two independent incubation experiments.

Figure 4.8 illustrates the reciprocity between IGF-1:IGFBP versus IGF-1: BI836845 complexes. This figure illustrates that the amount of BI836845 required for equal distribution of ligand between IGFBPs and the therapeutic antibody complexes is less than 33 nM, which corresponds to the amount of IGFBP-3 in serum. This indicates that, in serum, the affinity of BI836845 for IGF-1 is in the same order of magnitude as that of IGFBP-3.

The serum concentration of IGF-2 was not assayed. In contrast to humans, serum IGF-2 levels drop in mice after birth and very low levels are observed throughout adulthood (22). Therefore, given the low abundance of this ligand in the mouse serum used herein, we deemed its importance to be minor in the context of the current experiments.

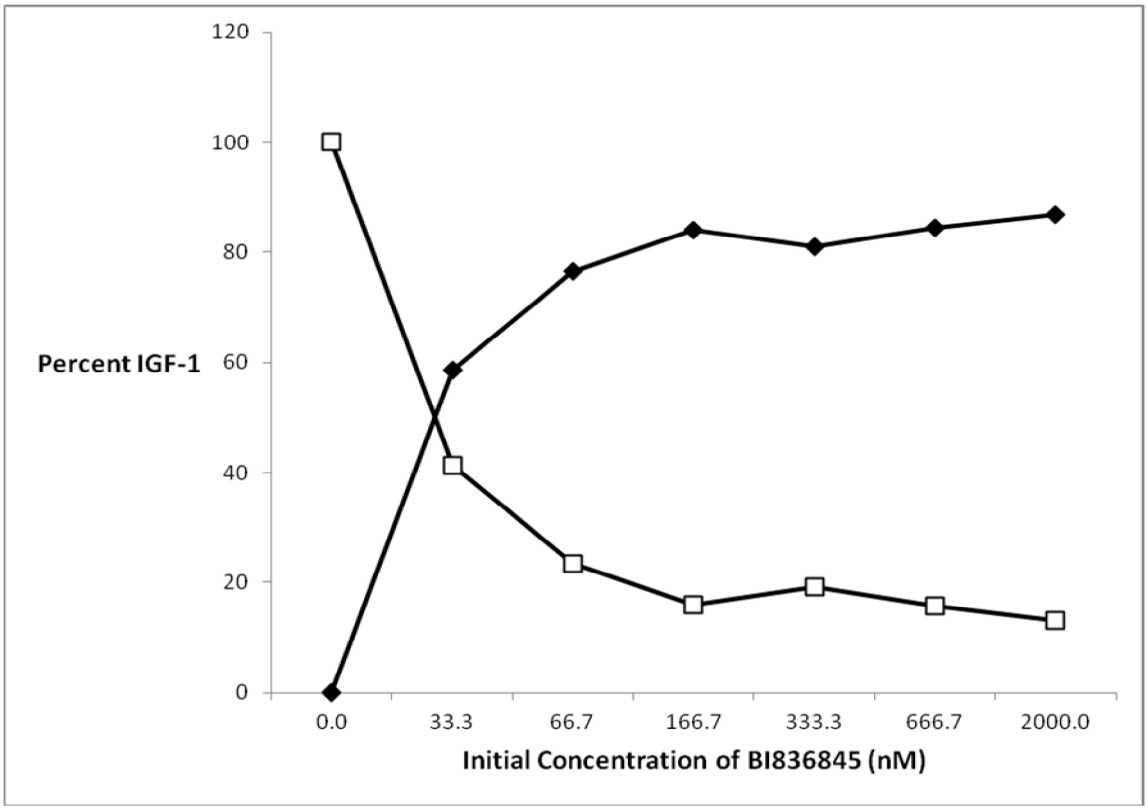
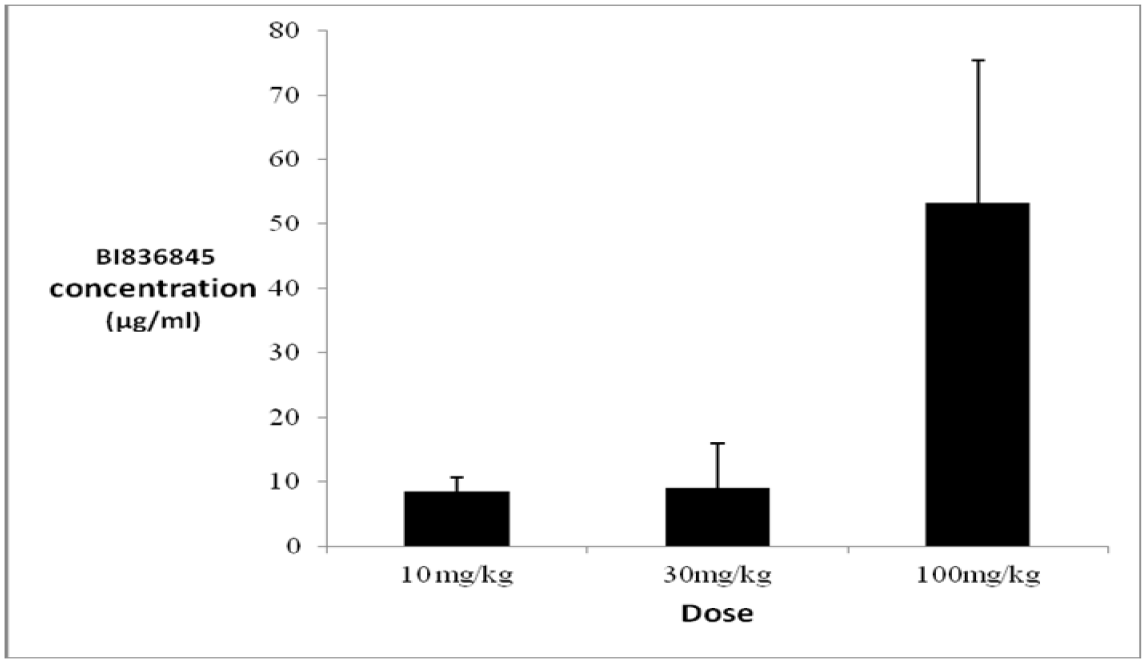


Figure 4.8: Schematic representation of results from Table 4.1 illustrating the reciprocity of IGF-1:IGFBP (white squares) or IGF-1: BI836845 (black squares) complexes. As the concentration of BI836845 increases, the proportion of IGF-1 bound to IGFBP-3 decreases. The concentration of IGFBP-3 in mouse serum is ~ 20 nM.

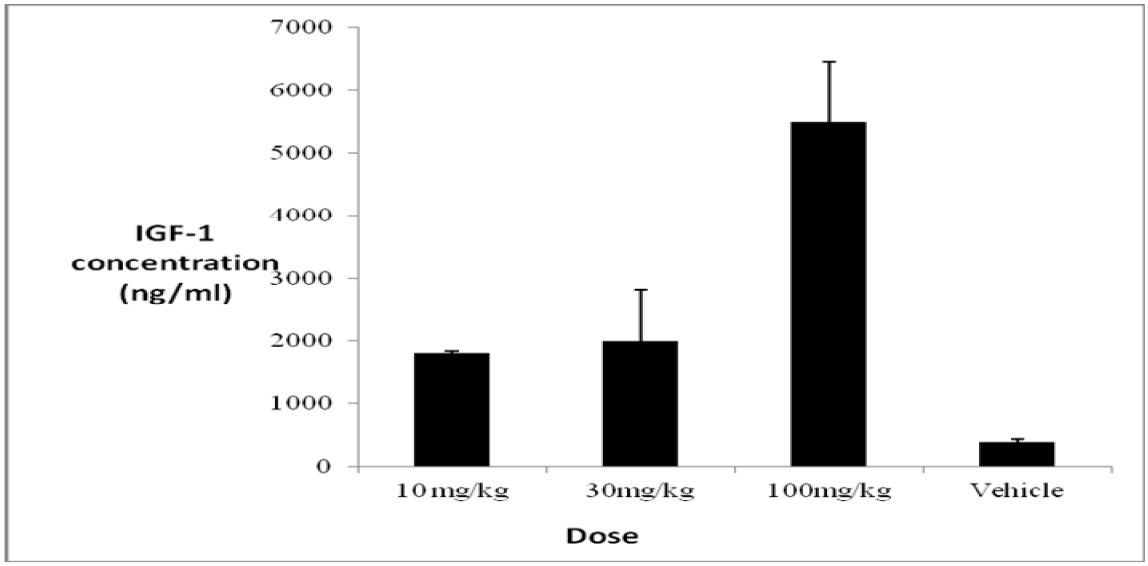
Effect of BI836845 on IGF-1 and IGFBP-3 in vivo

In order to investigate the effect of BI836845 on IGF-1 levels in vivo, we treated groups of C57 BL/6 mice with various doses of the therapeutic antibody for two weeks, following which animals were sacrificed four days after the last injection. Figure 4.9A shows that the concentration of BI836845 achieved in serum is dose dependent. Figure 4.9B illustrates that total levels of serum IGF-1 increase substantially, as previously shown (15) and figure 4.9C illustrates that the concentration of IGF-1 not complexed to BI836845 is similar to levels of IGF-1 in control mice. From this experiment, we concluded that although total levels of IGF-1 rise, the vast majority is complexed to the therapeutic antibody. However, levels of potentially bioactive IGF-1 (as estimated by the concentration of IGF-1 not complexed to BI836845) as well as total levels of IGFBP-3 (data not shown) are identical to concentrations observed in control mouse serum. Therefore, this set of data did not provide evidence for either an increase in free IGFBP-3 nor for a decrease in IGF-1 bioactivity following BI836845 treatment, despite prior evidence for reduced IGF-1 bioactivity in terms of endpoints such as somatic growth (15).

A)



B)



C)

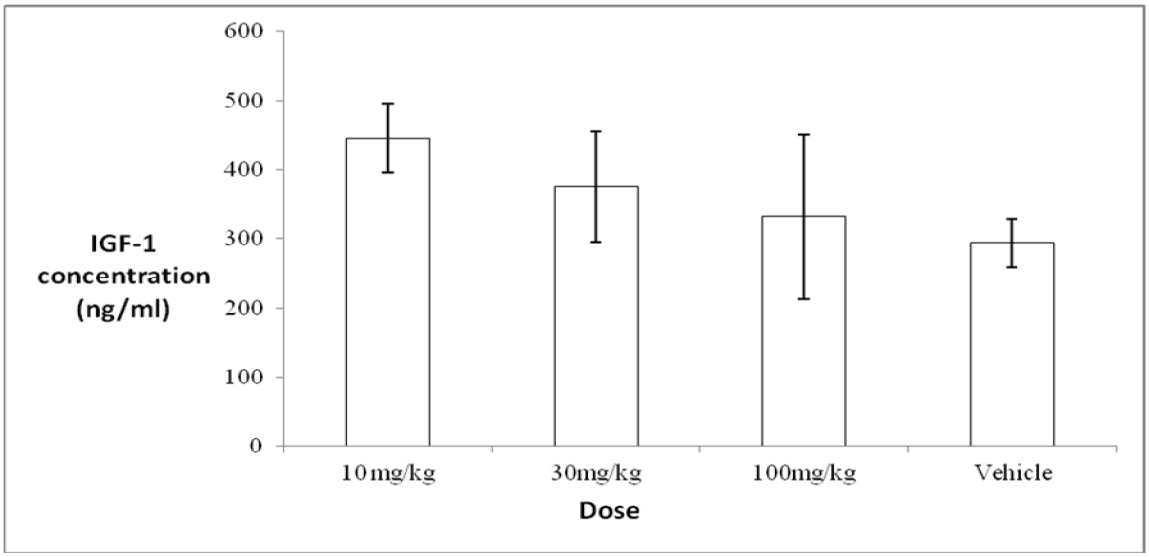
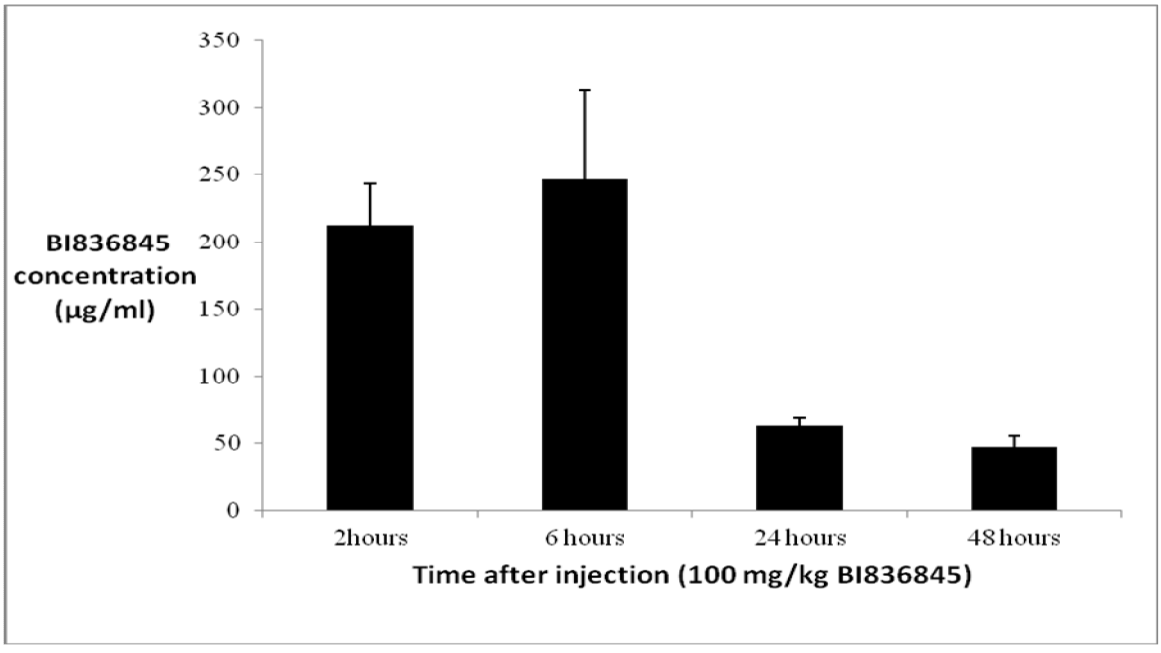


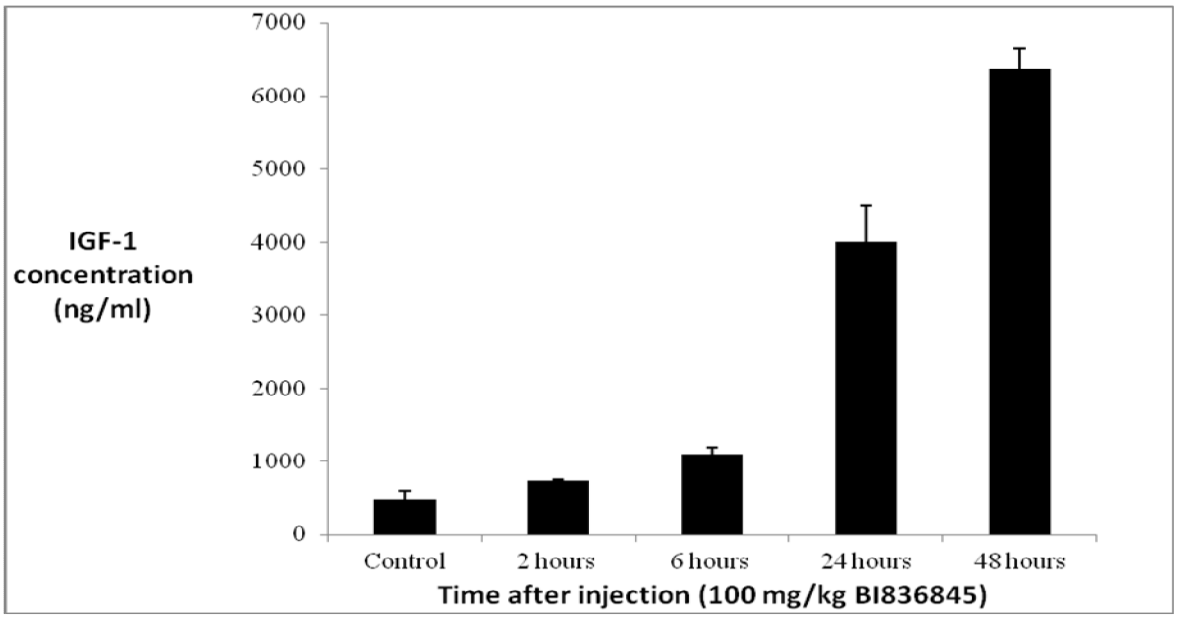
Figure 4.9: Mouse serum levels of BI836845, total IGF-1 and potentially bioactive IGF-1 (i.e. non-BI836845 complexed IGF-1). A) BI836845 concentration assayed by ELISA in serum from C57 BL/6 mice treated with indicated doses of BI836845 for two weeks, administered twice weekly, and sacrificed 4 days after the last injections. B) IGF-1 concentration in the same sera as in A). C) IGF-1 concentration in the flow-through fraction after Protein-A agarose column separation of sera used for A). In all panels error bars represent standard deviation of measurements from n=3 mice except for vehicle value, where n=4.

We next examined the effect of 100 mg/kg BI836845 treatment short term. C57 BL/6 mice were treated with BI836845 and then sacrificed at several time points over a two day period. Figure 4.10A shows that serum levels of antibody greater than 200 µg /ml are achieved after treatment but that these levels are maintained for less than 24 hours. Importantly, these antibody levels (1400 nM) are in vast molar excess of IGF-1 (80 nM). Figure 4.10B indicates that a 10 fold increase in total IGF-1 concentration occurs over 48 hours. Interestingly, figure 4.10C shows that levels of IGF-1 uncomplexed to BI836845 are lower than total IGF-1 levels in control mice for a period of at least 48 hours, which is consistent with the decrease in IGF-1 bioactivity previously reported (15). The amount of bioactive IGF-1 is lowest 6 hours after administration and gradually increases with time approaching control levels after 48 hours. Figure 4.10D illustrates a timeline of serum levels of both BI836845 and IGF-1 in molar terms. At earlier time points, there is a vast excess of binding sites over ligand, presumably leading to an initial decrease in bioavailable IGF-1, which is then gradually attenuated as ligand and binding sites become equimolar.

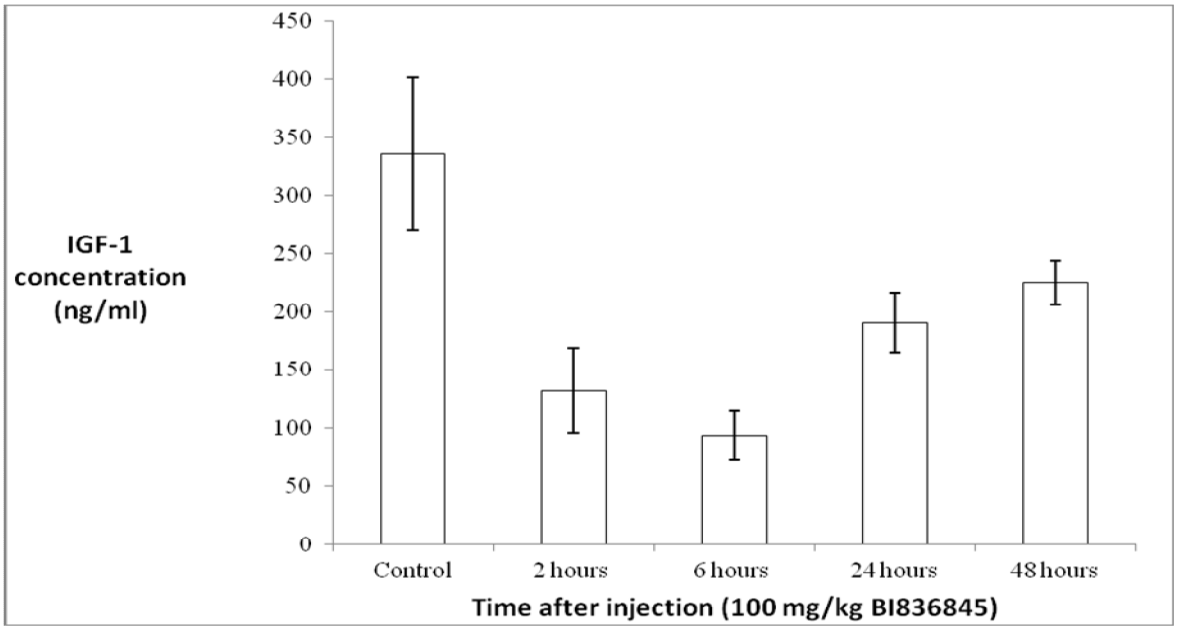
A)



B)


IGF-1 concentration (ng/ml)
7000
6000
5000
4000
3000
2000
1000
0
Control
2 hours
6 hours
24 hours
48 hours
Time after injection (100 mg/kg BI836845)

C)


IGF-1 concentration (ng/ml)
450
400
350
300
250
200
150
100
50
0
Control
2 hours
6 hours
24 hours
48 hours
Time after injection (100 mg/kg BI836845)

D)

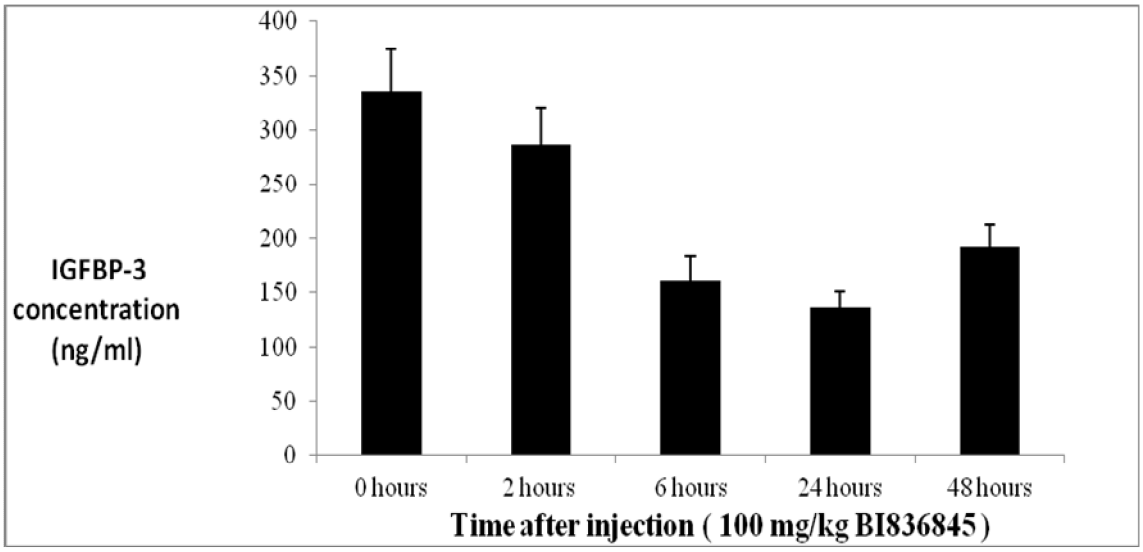
Time (hours)	Serum BI836845 (nM)	IGF-1 binding sites on BI836845 (nM)	Total Serum IGF-1 (nM)
0	0	0	61
2	1416	2832	94
6	1645	3290	142
24	418	836	520
48	318	636	828

Figure 4.10: Mouse serum levels of BI836845, total IGF-1 and potentially bioactive IGF-1. A) BI836845 concentration assayed by ELISA in serum from C57 BL/6 mice treated once with 100 mg/kg BI836845 and sacrificed at various time points over 48 hours. B) IGF-1 concentration in the same sera as in A). C) IGF-1 concentration in the flow-through fraction after Protein-A agarose column separation of sera used for A). D) Data from A), B) and C) represented in molar form. Binding sites are obtained by a doubling of BI836845 concentrations since two binding sites per antibody are present. In all panels error bars represent standard deviation of measurements from n=3 mice except for control value, where n=2.

Figure 4.11A shows a decline in total IGFBP-3 levels that is of similar magnitude to the decrease observed in bioavailable IGF-1 (figure 4.10C). Therefore, this set of data does not suggest that BI836845 leads to an increase in free IGFBP-3 in vivo, in contrast to our in vitro findings (figures 4.5 and 4.6).

Finally, figure 4.11B illustrates changes in growth hormone induced by BI836845 administration, suggesting that a pituitary compensation as a consequence of BI836845-induced decline in IGF-1 activity does occur, as previously reported following treatment with IGF-1R specific antibodies (13).

A)



B)

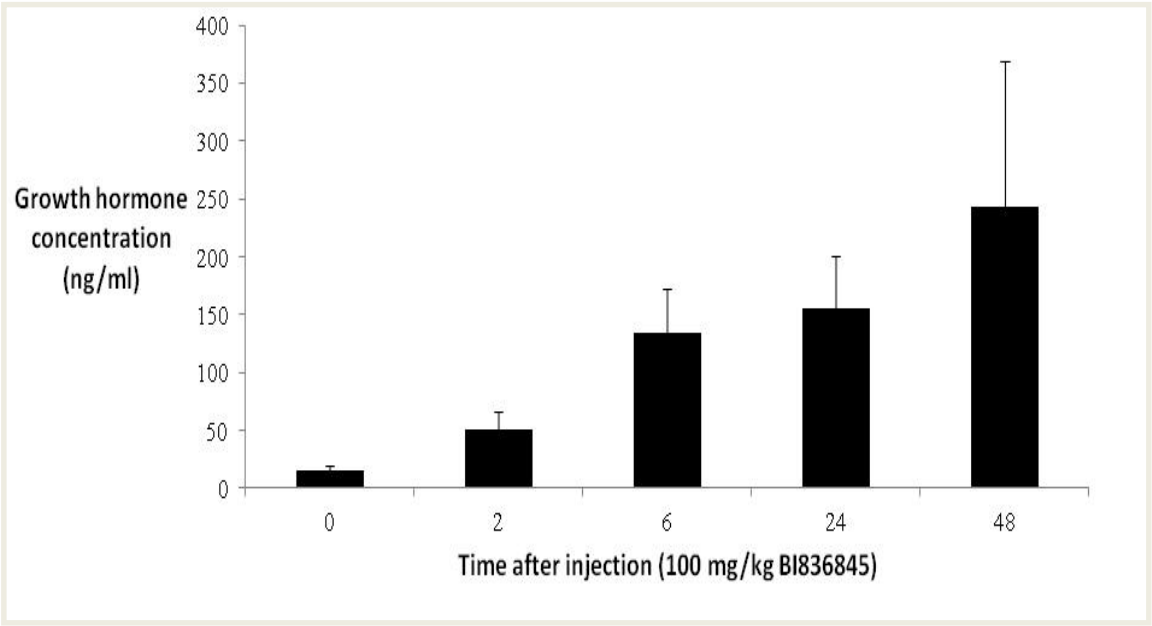


Figure 4.11: Mouse serum levels of IGFBP-3 and growth hormone. A) IGFBP-3 concentration assayed by ELISA in plasma from C57 BL/6 mice treated once with 100 mg/kg BI836845 and sacrificed at various time points over 48 hours. B) Growth hormone concentration in the same plasma samples as in A). In all panels error bars represent standard deviation of measurements from n=16 mice for the 0 time point, n=12 for the 2 hours time point, n=9 for the 6 hours time point, n=6 for the 24 hours time point, n=3 for the 48 hours time point.

4.5 Discussion

The human IgG1 antibody BI836845 is a novel candidate therapeutic agent for the treatment of cancers that are growth-stimulated by IGFs, which may be endocrine, paracrine or autocrine in nature. It represents a relatively novel strategy for targeting the IGF-1 axis by a ligand binding approach rather than an anti-receptor approach (such as figitumumab (23)) or small receptor tyrosine kinases inhibitors (such as BMS-554417 (24)). In vitro, it has been shown that BI836845 has a strong affinity for both human and rodent IGFs and that it inhibits IGF-1R phosphorylation in multiple cell line models (15). In rodent studies, BI836845 significantly inhibits growth of xenografted tumors, but also leads to large increases in total serum IGF-1 concentrations (15). The mechanisms responsible for this increase in total IGF levels and the impacts of BI836845 on IGF physiology as a whole are poorly described. However, in the light of this increase in ligand levels, it was of interest to quantify IGF-1: BI836845 complexes in serum. Although the original therapeutic goal of BI836845 was to reduce IGF-1 receptor activation, this drug candidate may also influence IGFBP physiology.

We developed a novel technique requiring minimal serum dilution to measure IGF-1:BI836845 complexes and applied it in a mouse model. We show that, in vitro, the addition of BI836845 shifts the binding of IGF-1 from IGFBPs to the therapeutic antibody. While IGF ligands complexed to IGFBPs can be released to “free” or “bioactive” ligands by physiological processes such as IGFBP proteolysis, the ligand bound to BI836845 is likely unavailable for receptor binding. Additionally, since IGFBP-3 has among the highest affinity for ligands, our results imply that other IGFBPs are also uncomplexed to ligand in the presence of BI836845. We also present evidence that the affinity of the therapeutic antibody in serum ex vivo for IGF-1 is comparable to that of IGFBP-3, which is in agreement with previous SPR studies conducted in simple buffer solutions. As BI836845 is currently in clinical trials, an extension of the technique

described in this report may be applicable in the quantification of IGF-1:BI836845 complexes directly in human serum.

Additionally, we confirm that administration of BI836845 in vivo leads to large increases in total IGF-1 levels, and we show that more than 90% of ligand is bound to the therapeutic antibody. The amount of IGF-1 unbound to BI836845, and therefore potentially bioactive, decreases below control levels after treatment. However, once most available binding sites of the antibody are occupied, bioactive IGF-1 levels revert to normal IGF-1 concentrations seen in untreated animals.

We also show that levels of growth hormone rise after BI836845 treatment in vivo, a finding that is consistent with a compensation mechanism due to loss of the inhibitory effect of IGF-1 on pituitary growth hormone secretion. A similar outcome has previously been observed following administration of IGF-1R specific antibodies (13). However, since we show that the vast majority of serum IGF-1 is bound to the therapeutic antibody, our data suggest that the presence of excess IGF-1 binding sites in serum is the major factor responsible for the observed rise in total IGF-1 levels. On the other hand, the observed increases in growth hormone may influence the rate at which binding sites become occupied or equivalently the rate at which total serum IGF-1 levels reach a maximum, which in turn is determined by BI836845 serum levels.

Interestingly, we have observed that the therapeutic antibody leads to increases in IGFBP-3 uncomplexed to ligand in mouse serum ex vivo. However, animal experiments did not indicate that a similar increase in free IGFBP-3 levels occurs in vivo since total levels of IGFBP-3 vary in the same direction and magnitude as levels of bioactive IGF-1. One possible explanation for this discrepancy is an increased serum clearance of free IGFBP-3 following BI836845 treatment. It was shown that ternary complexes of IGFBP-3:IGF-1:ALS are confined to the circulation as opposed to binary ligand complexes which readily cross the endothelial barrier due to smaller size (25). Therefore, it is possible that newly generated free IGFBP-3 is able to diffuse out of the circulation and into tissues.

This idea is attractive from a therapeutic point of view since it has been shown that IGFBP-3 can act independently of IGFs to inhibit cell growth and to increase apoptosis in a variety of models (26-28). A second possibility to account for the decrease in serum IGFBP-3 levels is a decreased liver output. In rodents, hepatic IGFBP-3 was reported to be positively regulated by both growth hormone and IGF-1 (29,30). We observed an increase in growth hormone levels following BI836845 administration as well as a simultaneous decrease in bioactive IGF-1. Therefore, the outcome of these opposing events requires further study. The possibility of BI836845 induced reduction of hepatocyte IGFBP-3 production via decrease in IGF-1R activation is under study using hepatocyte cultures.

Finally, we have observed that, at least in C57 BL/6 mice, BI836845 serum clearance is high and more than 75 % of initially achieved serum levels are eliminated within 24 hours of treatment. This finding may be explained by an immune response due to the non-murine nature of the antibody or it may be a species peculiarity. Therefore, it is likely that our murine experimental system does not accurately model BI836845 pharmacokinetics in humans. As serum samples from clinical trial subjects will be available in the near future, the techniques and models described in this report represent a useful framework for the analysis of the effects of BI836845 in humans.

Chapter References

1. Laviola L, Natalicchio A, Giorgino F. The IGF-I signaling pathway. Curr Pharm Des 2007;13:663-9.

2. Pollak MN, Schernhammer ES, Hankinson SE. Insulin-like growth factors and neoplasia. Nat Rev Cancer 2004;4:505-18.

3. Collett-Solberg PF, Cohen P. Genetics, chemistry, and function of the IGF/IGFBP system. Endocrine 2000;12:121-36.

4. Vincent AM, Feldman EL. Control of cell survival by IGF signaling pathways. Growth Horm IGF Res 2002;12:193-7.

5. LeRoith D, Roberts CT, Jr. The insulin-like growth factor system and cancer. Cancer Lett 2003;195:127-37.

6. Ghosh P, Dahms NM, Kornfeld S. Mannose 6-phosphate receptors: new twists in the tale. Nat Rev Mol Cell Biol 2003;4:202-12.

7. Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev 2002;23:824-54.

8. Boisclair YR, Rhoads RP, Ueki I, Wang J, Ooi GT. The acid-labile subunit (ALS) of the 150 kDa IGF-binding protein complex: an important but forgotten component of the circulating IGF system. J Endocrinol 2001;170:63-70.

9. Baserga R, Peruzzi F, Reiss K. The IGF-1 receptor in cancer biology. Int J Cancer 2003;107:873-7.

10. Resnicoff M, Sell C, Rubini M, Coppola D, Ambrose D, Baserga R, et al. Rat glioblastoma cells expressing an antisense RNA to the insulin-like growth factor-1 (IGF-1) receptor are nontumorigenic and induce regression of wild-type tumors. Cancer Res 1994;54:2218-22.

11. Reiss K, D'Ambrosio C, Tu X, Tu C, Baserga R. Inhibition of tumor growth by a dominant negative mutant of the insulin-like growth factor I receptor with a bystander effect. Clin Cancer Res 1998;4 :2647-55.

12. Sachdev D, Li SL, Hartell JS, Fujita-Yamaguchi Y, Miller JS, Yee D. A chimeric humanized single-chain antibody against the type I insulin-like growth factor (IGF) receptor renders breast cancer cells refractory to the mitogenic effects of IGF-I. Cancer Res 2003;63:627-35.

13. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. Nat Rev Cancer 2008;8:915-28.

14. Basu B, Olmos D, De Bono JS. Targeting IGF-1R: throwing out the baby with the bathwater? Br J Cancer 2011;104:1-3.

15. Adam PJ, Ostermann E, Lamche HR, Hofmann MH, Kroez M, Borges E, et al. Pharmacodynamic properties and antitumor efficacy of BI 836845, a fully human IGF ligand neutralizing antibody. Proccedings of AACR-NCI-EORTC International Conference: Molecular Targets and Cancer Therapeutics 2011;2011 Nov 12-16; San Francisco, CA.:Philadelphia (PA): AACR; Mol Cancer Ther 2011; 10(11 Suppl)-Abstr nr A208.

16. Gao J, Chesebrough JW, Cartlidge SA, Ricketts SA, Incognito L, Veldman-Jones M, et al. Dual IGF-I/II-neutralizing antibody MEDI-573 potently inhibits IGF signaling and tumor growth. Cancer Res 2011;71:1029-40.

17. Akerstrom B, Brodin T, Reis K, Bjorck L. Protein G: a powerful tool for binding and detection of monoclonal and polyclonal antibodies. J Immunol 1985;135:2589-92.

18. Bendayan M, Garzon S. Protein G-gold complex: comparative evaluation with protein A-gold for high-resolution immunocytochemistry. J Histochem Cytochem 1988;36:597-607.

19. Donovan SM, Hintz RL, Rosenfeld RG. Insulin-like growth factors I and II and their binding proteins in human milk: effect of heat treatment on IGF and IGF binding protein stability. J Pediatr Gastroenterol Nutr 1991;13:242-53.

20. Vermeer AW, Norde W. The thermal stability of immunoglobulin: unfolding and aggregation of a multi-domain protein. Biophys J 2000;78:394-404.

21. Beattie J, Phillips K, Shand JH, Szymanowska M, Flint DJ, Allan GJ. Molecular interactions in the insulin-like growth factor (IGF) axis: a surface plasmon resonance (SPR) based biosensor study. Mol Cell Biochem 2008;307:221-36.

22. Weber M, Milligan L, Delalbre A, Antoine E, Brunel C, Cathala G, et al. Extensive tissue-specific variation of allelic methylation in the Igf2 gene during mouse fetal development: relation to expression and imprinting. Mech Dev 2001;101:133-41.

23. Olmos D, Postel-Vinay S, Molife LR, Okuno SH, Schuetze SM, Paccagnella ML, et al. Safety, pharmacokinetics, and preliminary activity of the anti-IGF-1R antibody figitumumab (CP-751,871) in patients with sarcoma and Ewing's sarcoma: a phase 1 expansion cohort study. Lancet Oncol 2010;11:129-35.

24. Haluska P, Carboni JM, Loegering DA, Lee FY, Wittman M, Saulnier MG, et al. In vitro and in vivo antitumor effects of the dual insulin-like growth factor-I/insulin receptor inhibitor, BMS-554417. Cancer Res 2006;66:362-71.

25. Payet LD, Firth SM, Baxter RC. The role of the acid-labile subunit in regulating insulin-like growth factor transport across human umbilical vein endothelial cell monolayers. J Clin Endocrinol Metab 2004;89:2382-9.

26. Ingermann AR, Yang YF, Han J, Mikami A, Garza AE, Mohanraj L, et al. Identification of a novel cell death receptor mediating IGFBP-3-induced anti-tumor effects in breast and prostate cancer. J Biol Chem 2010;285:30233-46.

27. Williams AC, Smartt H, Zadeh AM, Macfarlane M, Paraskeva C, Collard TJ. Insulin-like growth factor binding protein 3 (IGFBP-3) potentiates TRAIL-induced apoptosis of human colorectal carcinoma cells through inhibition of NF-kappaB. Cell Death Differ 2007;14:137-45.

28. Massoner P, Colleselli D, Matscheski A, Pircher H, Geley S, Jansen DP, et al. Novel mechanism of IGF-binding protein-3 action on prostate cancer cells: inhibition of proliferation, adhesion, and motility. Endocr Relat Cancer 2009;16:795-808.

29. Clemmons DR, Thissen JP, Maes M, Ketelslegers JM, Underwood LE. Insulin-like growth factor-I (IGF-I) infusion into hypophysectomized or protein-deprived rats induces specific IGF binding proteins in serum. Endocrinol 1989;125:2967-72.

30. Glasscock GF, Hein AN, Miller JA, Hintz RL, Rosenfeld RG. Effects of continuous infusion of insulin-like growth factor I and II, alone and in combination with thyroxine or growth hormone, on the neonatal hypophysectomized rat. Endocrinol 1992;130:203-10.

General Discussion and Conclusion

Taken together, the studies described in this thesis reveal new facets of IGFBP physiology in the context of neoplasia. In the first part, we explored the regulation of IGFBP-2 in a breast cancer cell line. At that time, IGFBP-2 was the focus of a substantial amount of research due to several conflicting reports regarding its role in cancer. In some models, IGFBP-2 was shown to potentiate actions of IGF-1 and in other models to act independently of ligands (cf. (1)). Additionally, several epidemiologic studies had observed overexpression of IGFBP-2 in many malignancies including breast (2-5). It thus was unclear if IGFBP-2 expression was physiologically contributing to or merely a marker of malignant behavior. In the context of its overexpression in neoplasia, we felt it was important to study the molecular mechanisms responsible for the observed increase in protein levels. The PI3K/AKT/mTOR pathway was chosen as a starting point for two reasons. First we had evidence, both from the literature (6) and from prior work in our lab (7), that this pathway plays a significant role in the regulation of IGFBP-2 at the protein level. Second, the PI3K/AKT/mTOR cascade is frequently over active in a variety of cancers and this fact possibly accounts for a significant part of the results observed in the previously cited epidemiology reports.

A key finding of our study was that activation of the PI3K/AKT/mTOR pathway leads to increased IGFBP-2 mRNA levels as a result of increases in gene transcription. On the other hand, inhibitors of the pathway led to decreases in gene transcription and the effects were attributed to a small region in the proximal promoter known to bind Sp1 transcription factor. We also show that the PI3K/AKT/mTOR pathway does not alter the total levels of Sp1, but leads to multiple posttranslational modifications of the transcription factor. Our report and others (8-11) have associated AKT activation and Sp1 mediated gene transcription and this new link was recently reviewed in the context of Sp transcription factors and cancer metabolism (12). However, the mechanism responsible for the regulation of Sp1 activity is complex as the protein can be modified in several ways (OglnAc, acetylation, phosphorylation and

SUMOylation) and at multiple residues (for example phosphorylation can occur at more than 50 residues). Additionally, further complexity arises from the organ specific control of Sp1. This was evidenced in our study by the fact that insulin treatment did not induce changes in Sp1 modifications, while it has been shown to elicit Sp1 phosphorylation in hepatic cells (13).

Another important finding was that rapamycin, a well-known inhibitor of mTOR, leads to an increase in Sp1 O-GlnAcylation and a decrease in serine phosphorylation. Initially, we observed a decreased IGFBP-2 promoter activity following rapamycin treatment and one of our first hypothesized explanations for this effect was a rapamycin induced translational repression of a relevant transcription factor. However, this was not supported by our observations. Furthermore, effects of rapamycin on the activity of transcription factors have also been documented by others. For example, the phosphorylation of the transcription factor TIF1A is influenced by rapamycin through protein phosphatase 2A, a target of mTOR. More recently, the promoter activation of VEGF was shown to be inhibited following rapamycin treatment in renal cancer cells. Since Sp1 had been linked to the transcription of the VEGF gene, the authors concluded that rapamycin mediated changes in Sp1 activity are plausible (14). The specific mechanisms underlying the rapamycin induced posttranslational modifications we observed remain, however, unknown. Therefore, an important next step may be to further investigate manipulations of the PI3K/AKT/mTOR pathway and considering Sp1 posttranslational modifications as an end-point. A second unanswered question relates to which residues of Sp1 are modified in this context. Such a study may be a lengthy process since Sp1 contains more than 50 putative modification sites but nonetheless it is an important step towards understanding the specific molecular links between the PI3K/AKT/mTOR pathway and Sp1 regulation.

Additionally, a limitation of our study is the use of only one model cell line throughout most experiments. Although our main goal was to use MCF-7 cells as a paradigm for the elucidation of a mechanism of IGFBP-2 regulation following

PI3K/AKT/mTOR pathway manipulation, other studies are necessary to examine this system in other contexts. It is important to understand if the mechanism we describe is particular to a subset of cells or universal across cell types.

In the second part of the thesis, we show that neither 2-DG nor glucose influence IGF-1:IGFBP-3 binding. As was mentioned in chapter 1, IGFBPs regulate IGF bioavailability through at least two mechanisms. Firstly, they extend the half-life of IGF-1 thereby creating a relatively stable pool of ligand. Secondly, they regulate IGF activity at the cell surface by competing with the IGF-1R for ligand binding. Due to these opposing effects, the outcome of small molecules interfering with IGF:IGFBP binding on whole organism physiology is unclear. Nevertheless, there is some interest in the development of such small compounds, and in some cases, studies have led to the development of potential candidates (15).

The motivational article for our study claimed that 2-DG acts as a small molecule inhibitor of IGF-1:IGFBP-3 complex formation and exposure would therefore lead to increases in IGF-1R and AKT signaling, which was observed by the authors in a variety of cell lines. This report, if true, not only would have had an impact in the area of inhibitors of IGF:IGFBP binding but also in the context of cancer treatment since glycolysis inhibition is considered a potential therapeutic strategy. We wanted to extend this report and further characterize the effects of 2-DG, and also glucose because of its analogous structure, on IGF-1:IGFBP-3 complex formation. Importantly, we were unable to observe the effects claimed by the authors. We used three different techniques to examine the binding of IGF-1 to IGFBP-3 and the presence of 2-DG had no effect. Additionally, we were unable to reproduce the effect of 2-DG on IGF-1R activation in cell lines used by the authors. On the contrary, we saw a consistent decline in IGF-1R phosphorylation and uncorrelated levels of AKT activation upon 2-DG treatment.

Even though our results were mainly disproving, it was important to report this given the implications of the previous study. The efficacy/safety of 2-DG as a cancer therapeutic is still under investigation and the possibility that 2-DG

exposure can lead to unexpected increases in IGF-1R activation had to be addressed. On the other hand, our results had some positive implications. First, we showed that 2-DG exposure actually decreases IGF-1R activation in a panel of cell lines which is in agreement with its use as growth inhibitor. Secondly, the fact that AKT phosphorylation levels upon 2-DG treatment were not consistent across cell lines or with IGF-1R activity is indicative that the underlying mechanisms are related to differences in intracellular signaling and may even be cell line specific. Finally, we showed for the first time that a novel mass spectrometry technique, which has so far been only validated for model protein interactions, is suitable for the study of IGFs and IGFBPs.

In the third part of the thesis, we show that a candidate therapeutic antibody with high affinity for both IGF-1 and IGF-2 leads to increases in free IGFBP-3 ex vivo. The antibody BI836845 is among a relatively novel category of agents that interfere with IGF-1 signaling. Other more broadly explored strategies consist of antibodies specific to the IGF-1R, for example figitumumab (Pfizer), or of receptor tyrosine kinase inhibitors such as BMS536924 (Bristol-Myers Squibb). Prior to the present study, little attention was given to the impact of anti-ligand antibodies on IGFBP physiology, as most focus was directed to effects on IGF-1R activation and related endpoints. In this line of research, there is a similar antibody manufactured by Astra Zeneca (MEDI-573) with some clinical success so far.

BI836845 was previously shown by SPR to have higher affinity than IGFBP-3 for both IGF-1 and IGF-2 and to lead to decreased levels of IGF-1R phosphorylation in a panel of cell lines. Additionally, treatment with the antibody was shown to reduce tumor growth in mouse xenograft studies. It was also shown to be particularly effective in model cell lines overexpressing IGFs. A significant subset of cancers is believed to rely on IGF autocrine loops (16) and BI836845 could prove particularly useful in carefully selected patients.

Key results of our study indicated that the antibody can generate free IGFBP-3 ex vivo but that this effect is not seen in vivo. A possible explanation to account for this finding is that free IGFBP-3 may escape into tissues due to its smaller size when unbound to IGF-1 and ALS. As mentioned in chapter 1, there is prior evidence suggesting that IGFBP-3 inhibits cellular proliferation and induces apoptosis in a variety of models. The possibility that this effect could contribute to the antineoplastic activity of BI836845 is an important unresolved question and represents a potential therapeutic advantage from a cancer therapy point of view.

Furthermore, we showed that BI836845 binds more than 90% of IGF-1 in vivo and that, depending on molar excess of antibody levels over ligand levels, BI836845 can lead to decreases in bioactive IGF-1. We also showed that growth hormone levels rise post BI836845 treatment consistent with a pituitary compensation mechanism. Given that the vast majority of IGF-1 is bound to BI836845 across all conditions, our data indicate that levels of antibody are the main determinant of total IGF-1 levels. We hypothesize that, although BI836845 serum concentration varies with time, at any given point the molar amount of antibody binding sites represents a ceiling for the molar amount of IGF-1 achievable in serum. We also hypothesize that the rate at which this maximum is achieved is determined by hepatic IGF-1 output, which in turn may be influenced by growth hormone levels.

Another important component of our study is the development of a robust technique to measure the amount of IGFs bound to the antibody. The determination of IGF-1:BI836845 complexes was important in order to fully assess the therapeutic potential of the candidate agent. Since BI836845 is also in clinical trials at the moment, human serum from treated patients will be available and the technique we developed could be used, after some modifications, to assess IGF/ BI836845 complex formation directly in humans.

Finally, our study shows that the degradation of serum BI836845 is initially very rapid in C57 BL/6 mice. This model may not accurately reflect human pharmacokinetics. As mentioned, BI836845 levels determine total and bioactive

IGF-1 levels, therefore pharmacokinetics of the antibody are an important consideration when choosing optimal dosing regimens. Our experiments suggest that such regimens should ideally continuously induce an antibody molar excess over ligand in serum. However, BI836845 clinical trial data will provide insight as to whether this is feasible, particularly in relationship to toxicity and patient convenience.

An immediate next step of our study is to measure serum concentrations of ALS and other IGFBPs in mice, in order to further characterize the effect of BI836845 on IGFBP physiology. Additionally, the measurement of liver IGF-1 and IGFBP-3 mRNA levels will be important in order to understand the impact of increased growth hormone concentrations on hepatic output of these proteins. These experiments will also provide further insight with respect to the decreased serum IGFBP-3 levels observed after BI836845 administration. It would be of particular interest to determine if decreased liver IGFBP-3 output explains the observed decreases in serum IGFBP-3.

We recognize that the C57 BL/6 mouse model has a further limitation. In rodents, IGF-2 is not found in adult animals and the presence of this ligand in human serum may give rise to further complexity. At the moment, it is assumed equivalent to and interchangeable with IGF-1 with respect to BI836845 binding. The validity of this assumption will be verified in future human studies.

Discussion References

1. Hoeflich A, Reisinger R, Lahm H, Kiess W, Blum WF, Kolb HJ, et al. Insulin-like growth factor-binding protein 2 in tumorigenesis: protector or promoter? Cancer Res 2001;61:8601-10.

2. Boulle N, Logie A, Gicquel C, Perin L, Le Bouc Y. Increased levels of insulin-like growth factor II (IGF-II) and IGF-binding protein-2 are associated with malignancy in sporadic adrenocortical tumors. J Clin Endocrinol Metab 1998;83:1713-20.

3. Busund LT, Richardsen E, Busund R, Ukkonen T, Bjornsen T, Busch C, et al. Significant expression of IGFBP2 in breast cancer compared with benign lesions. J Clin Pathol 2005;58:361-6.

4. Fottner C, Sattarova S, Hoffmann K, Spottl G, Weber MM. Elevated serum levels of IGF-binding protein 2 in patients with non-seminomatous germ cell cancer: correlation with tumor markers alpha-fetoprotein and human chorionic gonadotropin. Eur J Endocrinol 2008;159:317-27.

5. McDonald KL, O'Sullivan MG, Parkinson JF, Shaw JM, Payne CA, Brewer JM, et al. IQGAP1 and IGFBP2: valuable biomarkers for determining prognosis in glioma patients. J Neuropathol Exp Neurol 2007;66:405-17.

6. Martin JL, Baxter RC. Expression of insulin-like growth factor binding protein-2 by MCF-7 breast cancer cells is regulated through the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin pathway. Endocrinol 2007;148:2532-41.

7. Levitt RJ, Georgescu MM, Pollak M. PTEN-induction in U251 glioma cells decreases the expression of insulin-like growth factor binding protein-2. Biochem Biophys Res Commun 2005;336:1056-61.

8. Jiang J, Gu J. Beta1,4-galactosyltransferase V A growth regulator in glioma. Methods Enzymol 2010;479:3-23.

9. Sroka IC, Nagle RB, Bowden GT. Membrane-type 1 matrix metalloproteinase is regulated by sp1 through the differential activation of AKT, JNK, and ERK pathways in human prostate tumor cells. Neoplasia 2007;9:406-17.

10. Tang SW, Yang TC, Lin WC, Chang WH, Wang CC, Lai MK, et al. Nicotinamide N-methyltransferase induces cellular invasion through activating matrix metalloproteinase-2 expression in clear cell renal cell carcinoma cells. Carcinogenesis 2011;32:138-45.

11. Jin HO, An S, Lee HC, Woo SH, Seo SK, Choe TB, et al. Hypoxic condition- and high cell density-induced expression of Redd1 is regulated by activation of hypoxia-inducible factor-1alpha and Sp1 through the phosphatidylinositol 3-kinase/Akt signaling pathway. Cell Signal 2007;19:1393-403.

12. Archer MC. Role of sp transcription factors in the regulation of cancer cell metabolism. Genes Cancer 2011;2:712-9.

13. Majumdar G, Harrington A, Hungerford J, Martinez-Hernandez A, Gerling IC, Raghow R, et al. Insulin dynamically regulates calmodulin gene expression by sequential o-glycosylation and phosphorylation of sp1 and its subcellular compartmentalization in liver cells. J Biol Chem 2006;281:3642-50.

14. Basu A, Banerjee P, Contreras AG, Flynn E, Pal S. Calcineurin inhibitor-induced and Ras-mediated overexpression of VEGF in renal cancer cells involves mTOR through the regulation of PRAS40. Plos One 2011;6:e23919.

15. Liu XJ, Xie Q, Zhu YF, Chen C, Ling N. Identification of a nonpeptide ligand that releases bioactive insulin-like growth factor-I from its binding protein complex. J Biol Chem 2001;276:32419-22.

16. Dwarakanath BS, Singh D, Banerji AK, Sarin R, Venkataramana NK, Jalali R, et al. Clinical studies for improving radiotherapy with 2-deoxy-D-glucose: present status and future prospects. J Cancer Res Ther 2009;5 Suppl 1:S21-S26.

17. Comprehensive molecular characterization of human colon and rectal cancer. Nature 2012;487:330-7.

Appendix

Published articles

Growth Factors, 2010; Early Online: 1–13

informa
healthcare

IGFBP-2 expression in MCF-7 cells is regulated by the PI3K/AKT/mTOR pathway through Sp1-induced increase in transcription

MATEI MIREUTA, ANDREW DARNEL, & MICHAEL POLLAK

Departments of Medicine and Oncology, Lady Davis Institute for Medical Research, Montreal SMBD Jewish General Hospital, and McGill University, Montreal, Quebec, Canada H3T 1E2

(*Received 7 October 2009; revised 1 March 2010; accepted 2 March 2010*)

Abstract
Insulin-like growth factor binding protein 2 (IGFBP-2) has been implicated in the pathophysiology of neoplasia. The PI3K/AKT/mTOR pathway has recently been shown to be a predominant regulator of IGFBP-2 at the protein level in MCF-7 breast cancer cells. However, there are gaps in knowledge with respect to the molecular mechanisms that underlie this regulation. Here, we show that the PI3K/AKT/mTOR pathway regulates IGFBP-2 protein levels by modulating IGFBP-2 mRNA abundance in MCF-7 cells. This change is achieved by regulating transcription through a critical region present in the first 200 bp upstream of the transcription initiation site where Sp1 transcription factor binds and drives transcription. IGF-1 treatment leads to increased nuclear abundance of Sp1 and increased IGFBP-2 mRNA and protein levels. Rapamycin and LY294002 induce a decline in Sp1 nuclear abundance and IGFBP-2 mRNA and protein levels. This work provides a mechanistic explanation for the observed effects of the PI3K/AKT/mTOR pathway on IGFBP-2 levels in MCF-7 cells.

Keywords: *IGFBP-2, PI3K, AKT, mTOR, mRNA, Sp1*

Introduction

Insulin-like growth factor binding protein 2 (IGFBP-2) is a member of the family of IGFBPs, of which IGFBP-1 through 6 have been relatively well characterized. Although they are expressed in many tissues, IGFBPs have classically been defined as carrier proteins for insulin-like growth factors (IGFs) in the blood. They are approximately 36 kDa proteins and share common structural motifs, with conserved N- and C-termini involved in IGF binding and a variable central L (linker)-domain containing several posttranslational modification sites. It is thought that IGFBPs function in the circulation to regulate IGF bioavailability and half-life. IGFBP-3 is the most abundant IGFBP in the blood, binding IGFs as a trimer with acid-labile subunit. Other IGFBPs are present at lower concentrations (Drop et al. 1992; Baxter 1993; Jones and Clemmons 1995; Hwa et al. 1999; Firth and Baxter 2002). However, IGFBP-2 is the most abundant IGFBP in other fluids such as cerebrospinal fluid (CSF) and seminal plasma (Chesik et al. 2007).

Despite their relatively well-characterized functions in the blood, IGFBPs have more obscure functions in the local microenvironment of cells. They can inhibit actions of IGF-1 and IGF-2 by binding and sequestering them away from the IGF-1 receptor. At the same time, they can potentiate IGF actions by possibly acting as chaperones and binding cell surface structures and bringing IGFs in close proximity to the IGF-1R. Furthermore, they can act independently of IGFs by putative mechanisms such as direct integrin binding (RGD domains) or possibly specific receptors

Correspondence: M. Pollak, Departments of Medicine and Oncology, Jewish General Hospital, 3755 Chemin Cote Ste-Catherine, Montreal, Quebec, Canada H3T 1E2. Tel: 514 340 8222. Ext. 5530. Fax: 514 340 8600. E-mail: michael.pollak@mcgill.ca

ISSN 0897-7194 print/ISSN 1029-2292 online
DOI: 10.3109/08977191003745472

(Hoeflich et al. 1998; Parker et al. 1998; Campbell et al. 1999; Mohan and Baylink 2002; Frommer et al. 2006; Perks et al. 2007). Further complexity arises from the fact that IGFBPs L-domain exhibits protease cleavage, glycosylation, and phosphorylation sites which serve to regulate their functions even further (Sommer et al. 1993; Yu et al. 1998; Okabe et al. 1999). Moreover, multiple-sized fragments released from IGFBP proteolysis have been reported to have biological significance (Lalou et al. 1996).

Depending on the experimental model used, IGFBP-2 has been reported, like other IGFBPs, to potentiate, inhibit or act independently of IGFs *in vitro* (Chen et al. 1994; Menouny et al. 1998; Hoeflich et al. 2000, 2001; Dunlap et al. 2007; So et al. 2008). Epidemiologic studies have shown IGFBP-2 to be upregulated at the protein level in many malignancies and to be positively correlated with tumor progression in many cancers, including breast (Zumkeller et al. 1993; Boulle et al. 1998; Richardsen et al. 2003; Busund et al. 2005; McDonald et al. 2007; Fottner et al. 2008). It is, however, unclear whether IGFBP-2 is merely a marker of malignant cell behavior or it contributes to tumor growth. As an initial step toward understanding the actions of IGFBP-2, in the context of its overexpression in neoplasia, it is important to understand pathways responsible for its regulation.

Several publications have examined the regulation of IGFBP-2 in different cell types and have reported modulation by factors including IGF-1, insulin, and steroids (Ernst and White 1996; Chan et al. 2001; Elminger et al. 2001; Han et al. 2006). The PI3K/AKT/mTOR pathway is one of the most upregulated pathways in neoplastic cells through mechanisms such as phosphatase and tensin homolog (PTEN) loss of function or PI3K activating mutations (Blume-Jensen and Hunter 2001). This pathway may be predominant in the regulation of IGFBP-2 at the protein level. Studies have shown that activation of the PI3K/AKT/mTOR pathway leads to overexpression of IGFBP-2 in experimental models, including the MCF-7 breast cancer cell line (Levitt et al. 2005; Martin and Baxter 2007). However, there are still major gaps in knowledge with respect to the molecular mechanisms employed to regulate IGFBP-2 protein levels. Our goal was to extend current knowledge of the IGFBP-2 regulation by the PI3K/AKT/mTOR pathway and to propose a mechanistic explanation for the observed effects at the protein level.

Materials and methods

Cell lines and materials

The MCF-7 and T47D cell lines were purchased from (American Type Culture Collection, Manassas, VA, USA) and cultured in standard RPMI medium supplemented with 10% fetal bovine serum and 20 μg/ml Gentamicin. IGF-1, epidermal growth factor (EGF) and insulin were purchased from Cell Sciences (Canton, MA, USA), LY294002, rapamycin, PD98059, and SB203580 were purchased from Calbiochem (San Diego, CA, USA). Mithramycin A was purchased from BIOMOL (Zandhoven, Belgium).

ELISA

Conditioned medium from MCF-7 cells was collected, diluted 30 times in RPMI serum-free medium and IGFBP-2 concentration was measured by ELISA. The ELISA components (antibodies, standards, etc.) were purchased from RnD Systems (Minneapolis, MN, USA) while ELISA 96-well plates were purchased from Costar (Lowell, MA, USA). Manipulations were performed according to the manufacturer's instructions. IGFBP-2 concentrations in conditioned media are not corrected for cell number, as the steady states reached under each treatment were independent of cell number. We believe that these steady states are reached as a consequence of the system achieving an equilibrium between secretion and degradation rates. The measurements are thus left as concentration per well.

RNA collection and Quantitative real-time PCR

RNA was collected from MCF-7 cells using TRizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Then, 5 μg of total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase and random primers (Invitrogen). To eliminate the RNA template, RNase H was added to the reaction in the final step. Then, total cDNA was purified from the reaction mixture using silica columns (Qiagen, Venlo, The Netherlands). Concentrations of the relatively pure cDNA were assessed using absorbance spectrometry (Nanodrop) and 500 ng of total cDNA were used downstream in the Quantitative real-time PCR (qRT-PCR) experiments. Taqman probe, enzyme mix, and 96-well plates were purchased from Applied Biosystems (Foster City, CA, USA) and qRT-PCR was performed on a Light Cycler. Results were compared to a standard curve of known amounts of IGFBP-2 DNA and analyzed with the manufacturer's software.

Luciferase constructs and Sp1 plasmids

Respectively, a 2.4 and 1.2 kbp region upstream of the IGFBP-2 translation start site were cloned into the PGL-3 luciferase enhancer vector (Promega, Madison, WI, USA). The 2.4 kbp region was cloned by PCR using forward primer: 5′-CGTTTGCGATTTGCAGTAGA-3′ and reverse primer: 5′-CTCCTCCGCTTCTTCCTCCT-3′ while the

1.2 kbp sequence was cloned using forward primer: 5′-GTGGAGGCAGCTTAATGGTC-3′ and reverse primer 5′-CTCCTCCGCTTCTTCCTCCT-3′. Bgl-2 and Kpn-1 sites were introduced into the primers and the amplification product was cloned utilizing the same restriction sites in the vector. The 700 and 200 bp sequences were generated using restriction enzymes Xho-1 and Sma-1 in the 1.2 kbp construct and were ligated at the same sites into the vector.

pPacSp1 vector contains a sequence coding for a truncated version of the transcription factor Sp1, which has been shown to maintain at least 90% of full-length Sp1 function (Courey and Tjian 1988). pPac0 is an empty vector and served as a control. Both plasmids were purchased from Addgene (Cambridge, MA, USA).

Cell lysates and nuclear/cytoplasmic fractionation

Total cell lysates were obtained using RIPA buffer as described previously (Levitt et al. 2005). Nuclear lysates were obtained using three buffers: Buffer A (50 mM NaCl, 10 mM HEPES, 500 mM sucrose, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 0.2% TX-100, and 7 mM mercaptoethanol), Buffer B (50 mM NaCl, 10 mM HEPES, 25% glycerol, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, and 7 mM mercaptoethanol), Buffer C (350 mM NaCl, 10 mM HEPES, 25% glycerol, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, and 7 mM mercaptoethanol) all supplemented with protease inhibitors. Briefly, cells were collected and lyzed with Buffer A for 2 min and then centrifuged. The supernatant was termed cytoplasmic extract. Subsequently, the precipitate was washed twice in Buffer B for 2 min and then it was lyzed using Buffer C on ice for 30 min. Final centrifugation yielded nuclear extracts.

Transfections, western blots, and luciferase measurements

Transfections were done using electroporation (Microporator, Digital Bio Technology, Seoul, Korea) and cells were cultured in RPMI medium containing serum for 24 h. Cells were then incubated in serum-free medium containing treatments and left in culture for 72 h until measurements were performed.

Luciferase measurements were done on a luminometer following the addition of Bright-GLO luciferase substrate solution (Promega) to the media according to the manufacturer's specifications.

For western blots, total protein from lysates was quantified (BioRad, Hercules, CA, USA). Thirty micrograms of total protein were loaded per lane and were further transferred onto a nitrocellulose membrane. These were immunoblotted with specific antibodies for Sp1, α-tubulin, lamin A/C (Santa Cruz, Santa Cruz, CA, USA), phospho-serine 473 AKT, AKT, p70 S6 Kinase, phospho-threonine 389 p70 S6 Kinase, and beta-actin (Cell Signaling, Danvers, MA, USA). Finally, membranes were exposed on chemiluminescence film.

Immunofluorescence

Cells were washed three times in PBS for 5 min each time, fixed with paraformaldehyde for 10 min and washed again three times in PBS. Then, cells were incubated for 30 min in PBS containing 0.5% BSA and 0.5% Triton X-100. Anti-Sp1 antibody was diluted 1:100 in PBS containing 0.5% BSA and 0.5% Triton-X and applied overnight in a humidified chamber. The following day, cells were washed three times in PBS and the fluorochrome-conjugated secondary antibody (Invitrogen) was added at a 1:1000 dilution in PBS containing BSA and Triton-X for 1 h. Cells were then washed three times in PBS and mounted with media containing 4',6-diamidino-2-phenylindole (DAPI) (Vectashield, Burlingame, CA, USA). The slides were visualized using a Leica microscope and an appropriate software.

Immunoprecipitation and chromatin immunoprecipitation

For immunoprecipitation of Sp1, cells were lyzed using RIPA buffer supplemented with protease and phosphatase inhibitors (Roche, Basel, Switzerland). Then, 500 μg of total cellular protein was incubated for 1 h with 40 μl protein A/G-conjugated beads (Santa Cruz) for preclearance at 4°C. Beads were removed by centrifugation and the lysates were further incubated with 1 μg Sp1-specific antibody (Santa Cruz) for 1 h at 4°C. Afterwards, 50 μl of protein A/G-conjugated agarose beads were added to each tube and incubated overnight on a rotating device at 4°C. The following day, the tubes were washed once in 1 ml of RIPA buffer supplemented with protease and phosphatase inhibitors (centrifugation at 9000 rpm for 1 min) and final resuspension was done in SDS-PAGE 6X concentrated loading buffer. Samples were boiled for 5–10 min and loaded on a SDS-PAGE gel. After transfer, nitrocellulose membranes were immunoblotted with specific antibodies for small ubiquitin-like modifier protein (SUMO) phospho-threonine, phospho-serine, phospho-tyrosine (Santa Cruz) and O-linked *N*-Acetylglucosamine (Pierce, Rockford, IL, USA).

For chromatin immunoprecipitation, cells were fixed in 1% formaldehyde for 25 min, washed three times in PBS and lyzed with RIPA buffer supplemented with protease and phosphatase inhibitors. Then, cells were sonicated on ice 12 times for 10 s each at 40% amplitude in a Sonic Dismembrator Model 500 (Fisher Scientific, Pittsburgh, PA, USA).

Furthermore, lysates were centrifuged at 13,000 rpm 4°C, and total protein levels in the supernatant were quantified. Three milligrams of total protein were incubated with 40 µl of protein A/G agarose (Santa Cruz) beads for preclearance for 2 h at 4°C in a rotating device. Beads were removed by centrifugation and the lysates were further incubated with 3 µg anti-Sp1 antibody for 1 h followed by the addition of 70 µl of protein A/G agarose beads and incubation overnight at 4°C in a rotating device. The following day, beads were washed in RIPA three times and resuspended in a buffer containing 50 mM TRIS pH 8, 1 mM EDTA, 1% SDS, 50 mM $NaHCO_3$, 200 mM NaCl, and 5 mM DTT, and incubated at 65°C overnight with shaking. The following day, 2 µl of a 10 mg/ml Proteinase K stock solution were added and the tubes were further incubated at 42°C for 2 h. Finally, the DNA was extracted with phenol/chloroform/isoamyl alcohol solution twice and used as the input template in a conventional PCR for 35 cycles. The primers used (left primer: 5′-AGCAGGGAACCCCCAGAG-3′ and right primer: 5′-CTAAAGGGCCGGCTTCTC-3′) are complementary to a region within 200 bp upstream of the IGFBP-2 transcription initiation site and produce a PCR product of 156 bp.

Results

IGFBP-2 protein and mRNA are regulated through the PI3K/AKT/mTOR pathway in MCF-7 breast cancer cells

As IGFBP-2 protein levels have been previously shown to be modulated by the PI3K/AKT/mTOR pathway in MCF-7 cells, we wished to investigate whether these effects also occur at the mRNA level. We treated MCF-7 cells with activators IGF-1, insulin, EGF, and inhibitors LY294002 (PI3K inhibitor) and rapamycin (mTOR inhibitor) at concentrations previously reported to affect IGFBP-2 protein levels. We confirm that IGF-1 (13 nM) induces an approximate threefold increase in IGFBP-2 protein levels in conditioned media of MCF-7 cells and LY294002 treatment (30 µM) results in a decrease of about sevenfold while rapamycin (100 nM) induces a twofold decrease. Insulin (172 nM) and EGF (171 nM), despite 100-fold higher concentrations, provoke more modest changes in IGFBP-2 protein levels (about 50% increase by insulin and no statistically significant increase by EGF) (Figure 1A). IGFBP-2 protein levels as well as mRNA levels were measured at three time points: 24, 48, and 72 h under all treatments. LY294002 and rapamycin treatment induced new steady states in both mRNA and protein levels after 24 h of treatment which did not change at the subsequent 48 and 72 h time points (data not shown). Similarly, IGF-1 treatment induced new steady states of IGFBP-2 mRNA and protein levels after 48 h of treatment (data not shown). Since our goal was to measure steady-state changes in IGFBP-2 mRNA and protein levels rather than short-term effects, all subsequent measurements were performed after 72 h of treatment, a time sufficient for cells to reach steady-state levels of expression under all treatments.

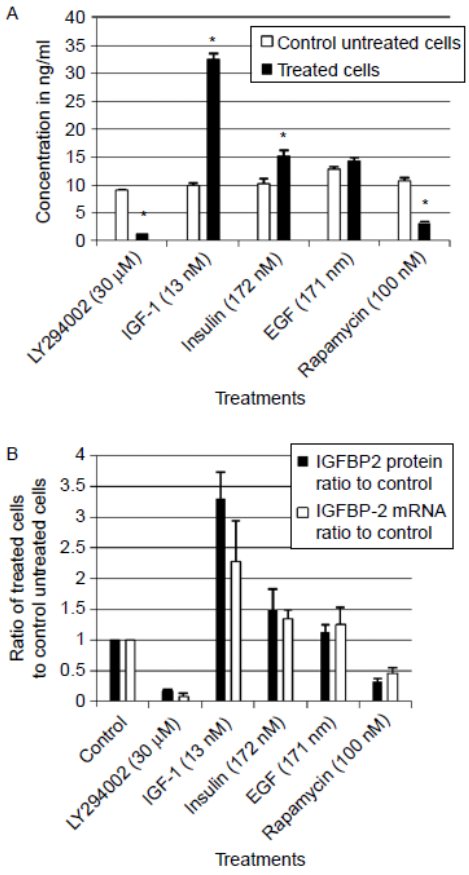


Figure 1. Effect of PI3K/AKT/mTOR pathway modulators on IGBP-2 protein and mRNA expression in MCF-7 breast cancer cells. (A) Concentration of IGFBP-2 protein in conditioned media of MCF-7 cells. Cells were plated at 5×10^5 cells per well in six-well plates and cultured for 24 h. The media were then exchanged for serum-free media containing the desired concentrations of treatments (IGF-1 at 13 nm, LY294002 at 30 µM, rapamycin at 100 nM, insulin at 172 nM, and EGF at 171 nM). Cells were further incubated for 72 h following which media were collected and IGFBP-2 concentration was measured by ELISA. Concentration is expressed as ng/ml per well. *indicates statistical significance compared to control as obtained by ANOVA ($n = 12$, p-value < 0.0001). (B) Ratios of IGFBP-2 mRNA and protein measurements compared to control untreated cells. Cells were cultured as above and IGFBP-2 mRNA was measured using qRT-PCR and is expressed as ratios of treated cells to control untreated cells. Protein measurements are the same as in (A), but expressed as ratios of treated cells to control untreated cells. Error bars indicate approximate 95% confidence intervals obtained by the delta-method approximation to Fieller's method with $n = 6$ for IGFBP-2 mRNA.

As shown in Figure 1B, IGFBP-2 mRNA, as measured by qRT-PCR, varies in the same direction and with similar magnitude as the relative protein changes induced by the above-mentioned treatments. IGF-1 treatment results in the highest induction of IGFBP-2 mRNA (~ twofold) while LY294002 and rapamycin decrease IGFBP-2 mRNA levels by roughly eight and twofolds, respectively. Insulin, as was the case for protein levels, induced a relatively small increase in mRNA levels (~25%), while EGF induced no statistically significant change.

Modulators of the PI3K/AKT/mTOR pathway act through DNA elements proximal to the transcription initiation site

In order to understand the effects of manipulation of the PI3K/AKT/mTOR pathway on IGFBP-2 mRNA regulation, we conducted a luciferase reporter experiment in order to isolate a region upstream of the IGFBP-2 transcription start site that might mediate the changes observed. We cloned several sequences proximal to the IGFBP-2 transcription start site, ranging from 2.4 kbp to 200 bp, using PCR on MCF-7 genomic DNA (Figure 2) directly upstream of a luciferase gene in the PGL-3 vector. MCF-7 cells transfected with the latter constructs were either treated or not with each modulator of the PI3K/AKT/mTOR pathway and 3 days later, the cells were lyzed and their respective luciferase expression was measured. The changes observed are expressed as ratios of treated transfected cells to untreated transfected cells for each construct for both the activators and inhibitors of the pathway (Figure 3A,B).

The change with each treatment correlates both in direction and magnitude with the change observed for IGFBP-2 mRNA levels, suggesting that these compounds affect mainly transcription of the IGFBP-2 gene rather than any other mechanism of mRNA regulation. As for IGFBP-2 mRNA levels, IGF-1 (13 nM) increases luciferase expression by roughly twofold whereas rapamycin (100 nM) and LY294002 (30 μM) decrease expression (1.5- and 5-folds, respectively). Insulin (172 nM) and EGF (171 nM) do not induce any change in luciferase expression. Also, none of the treatments have an effect on the Promoterless construct nor on the 1.2 kbp Control construct, indicating that the effects observed are not due to nonspecific factors such as increased cell death or luciferase translational modulation. In absolute values, the Promoterless and 1.2 Control constructs displayed around 10% luminescence activity compared to the remaining three constructs and green fluorescent protein (GFP) transfected or untransfected cells displayed no luminescence activity (data not shown). Additionally, since the same change is present across all constructs for all treatments, the data suggest that the critical region present in the first 200 bp upstream of the transcription initiation site is responsible for the effects observed on the IGFBP-2 mRNA.

IGF-1, LY294002, and rapamycin modulate IGFBP-2 mRNA levels through Sp1

It has been previously shown for the rat IGFBP-2 gene that Sp1 or Sp1-like elements bind to four guanine-cytosine (GC) rich boxes situated in the proximal

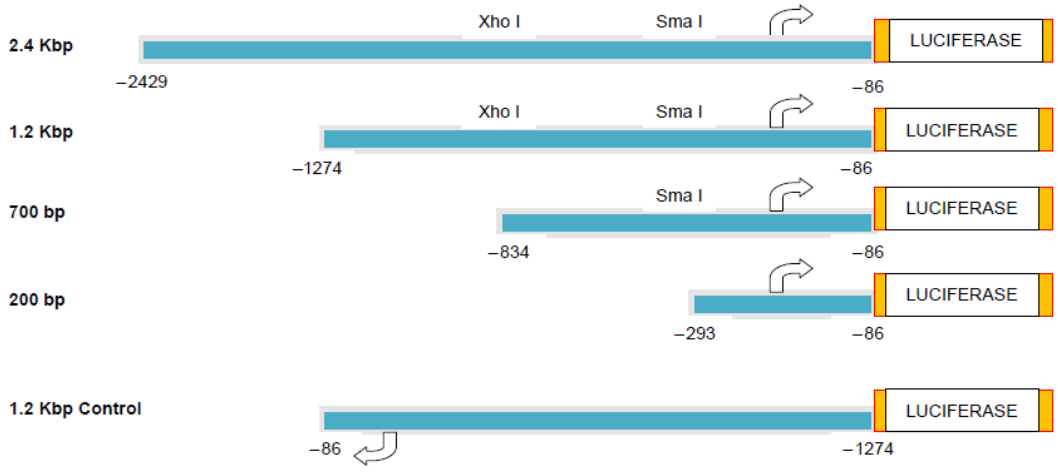


Figure 2. Schematic diagram of luciferase reporter assay constructs. A 2.4 and 1.2 kbp sequence upstream of the translation initiation site were cloned into the PGL-3 vector. Positions relative to the first ATG of IGFBP-2 are indicated below, the arrow represents the IGFBP-2 transcription initiation site. The 1.2 kbp Control construct was used as a negative control and represents the same sequence as the 1.2 kbp construct but cloned in the reverse orientation.

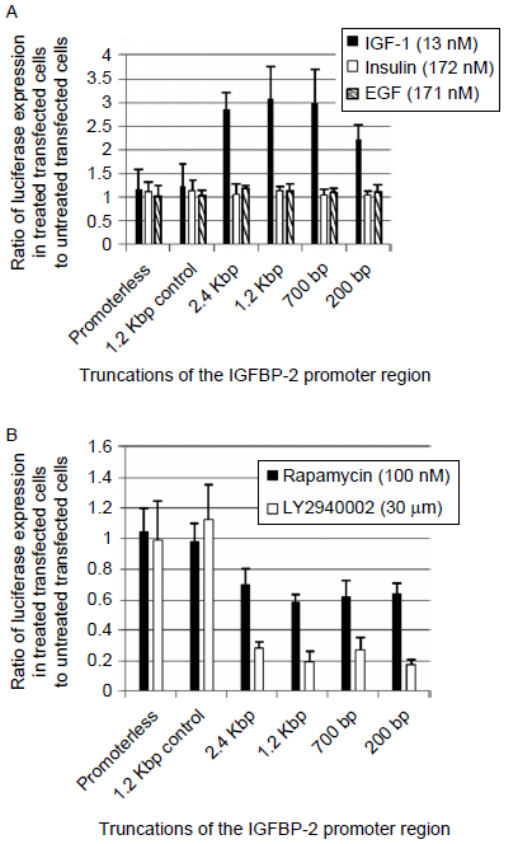


Figure 3. Effect of PI3K/AKT/mTOR pathway modulators on serially truncated IGFBP-2 promoter region sequences. (A) Effect of activators of the pathway. 4×10^5 MCF-7 cells were transfected with 2 μg of DNA per well in 24-well plates using electroporation. Cells were cultured for 24 h and then transferred to serum-free conditions with and without the appropriate growth factors. Luciferase readings were performed 72 h (steady state) later. Ratios shown are luciferase readings of treated transfected cells to untreated transfected cells for each construct. Error bars represent approximate 95% confidence intervals obtained by the delta-method approximation to Fieller's method with $n = 6$. (B) Effect of inhibitors of the pathway. Cells were treated exactly as in (A).

promoter region to drive transcription, although the study did not consider effects of any regulators of expression (Boisclair et al. 1993). Since these four boxes are almost identical in humans, and present in our 200 bp construct, we hypothesized that Sp1 transcription factor could be modulated by the PI3K/AKT/mTOR pathway in a way that could explain our observations.

We overexpressed Sp1 in MCF-7 cells along with the 200 bp sequence construct. Cotransfection of the 200 bp construct with an Sp1 plasmid results in an increase in luciferase expression whereas cotransfection with a control plasmid does not induce any change. Also, the increase produced by Sp1 is abrogated when cells are treated with Mithramycin A (500 nM), a compound which binds GC-rich regions and inhibits Sp1-driven transcription (Gingras et al. 2004) (Figure 4A).

Furthermore, we performed a chromatin immunoprecipitation experiment in order to investigate the levels of Sp1 transcription factor bound to the region comprising the four GC-rich boxes upstream of the IGFBP-2 transcription initiation site. We performed, in MCF-7 cells, an immunoprecipitation of total Sp1 cross-linked DNA followed by PCR amplification of the sequence containing the four GC-rich boxes. Compared to samples from control untreated cells, samples from IGF-1 (13 nM)-treated cells displayed higher levels of PCR product, while samples from LY294002 (30 μM) and rapamycin (100 nM)-treated cells displayed smaller levels of PCR product. Samples from insulin (172 nM) and EGF (171 nM) treated cells led to no apparent change in the levels of PCR product as compared to control.

Nevertheless, none of the previous treatments affected Sp1 protein abundance in total cell extracts (Figure 5A). The normal functioning of Sp1, like that of many other transcription factors, requires shuttling of the transcription factor between the cytoplasm and nucleus. We, therefore, fractionated cells into nuclear and cytoplasmic extracts followed by immunoblotting (Figure 5B). Our results indicated that IGF-1 (13 nM) increases the nuclear proportion of Sp1, whereas LY294002 (30 μM) and rapamycin (100 nM) decrease it. Densitometry measurements indicated that the changes in nuclear Sp1 are correlated with those of luciferase expression and IGFBP-2 mRNA. IGF-1 increased Sp1 nuclear abundance by around 80% while rapamycin and LY294002 decreased it by 22 and 80%, respectively. Insulin (172 nM) and EGF (171 nM) treatments did not result in significant increase in nuclear Sp1 (Figure 5C,D).

Furthermore, we conducted an immunofluorescence experiment with MCF-7 cells in order to visualize the large changes in nuclear Sp1 abundance after exposure to IGF-1 or LY294002 and observed that nuclear Sp1 increases with IGF-1 exposure and decreases with LY294002 exposure, consistent with western blot results (Figure 6A). The magnitude of changes seen with rapamycin exposure by western blot was too small to be detected by immunofluorescence and no effect on nuclear Sp1 by insulin nor EGF was observed (data not shown). Similar results were observed with the breast cancer cell line T47D which was previously shown to regulate IGFBP-2 protein levels through the PI3K/AKT/mTOR pathway (Martin and Baxter 2007) (Figure 6B).

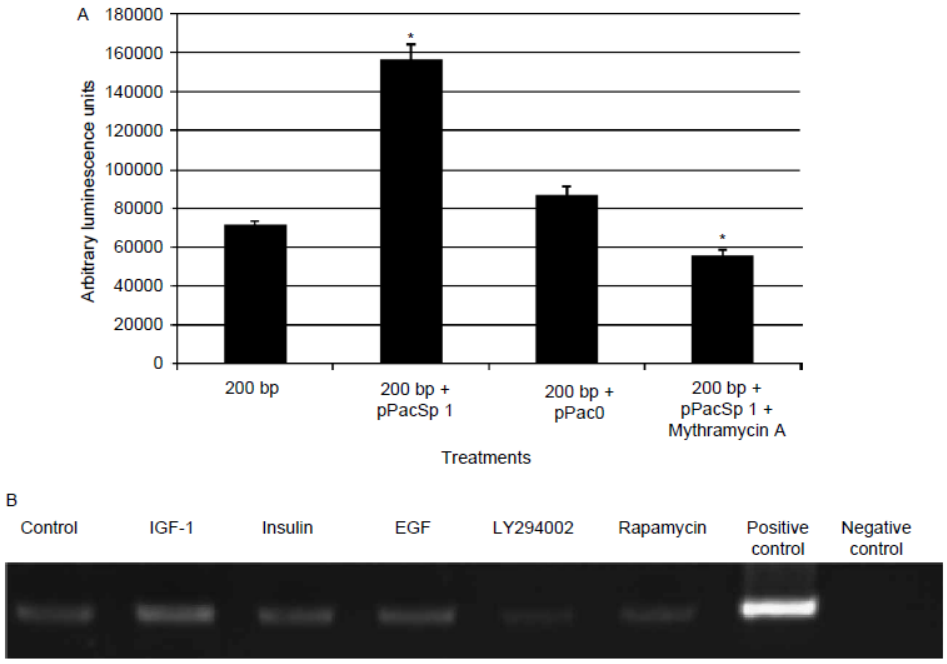


Figure 4. Effects of Sp1 on IGFBP-2 promoter transactivation. (A) The effect of overexpression of Sp1 on the 200 bp luciferase reporter construct. 4×10^5 cells were transfected with 2 μg of 200 bp construct or cotransfected with the former and 2 μg of pPac Sp1 or pPac 0 vector (Addgene) accordingly. Cells were left to incubate for 24 h and transferred to serum-free media with or without Mithramycin A (an Sp1 inhibitor) at 500 nM for an additional 24 h before luciferase measurements were performed. Error bars are the SEM and * represents statistical significance compared to control as obtained by ANOVA ($n = 6$, p-value < 0.001). (B) Chromatin immunoprecipitation. Cells were cultured in 10% fetal bovine serum (FBS) media in 15 mm round dishes until 80% confluence was achieved, then treated in serum-free media for 72 h with the different compounds. A chromatin immunoprecipitation experiment was carried out and the product was amplified by PCR. The lane termed positive control represents a PCR with the 700 bp construct as a template and the lane termed negative control represents a PCR on a sample from control untreated cells without anti-Sp1 primary antibody. The gel shown is representative of three independent experiments.

IGF-1, Rapamycin, LY294002 lead to changes in Sp1 posttranslational modifications in MCF-7 cells

In order to understand the regulation of Sp1 nuclear abundance by the different treatments examined so far, we investigated their effect on five posttranslational modifications of Sp1 known to possibly affect its nuclear abundance (Solomon et al. 2008): levels of total serine, tyrosine, threonine phosphorylation, SUMOylation, and O-linked *N*-acetylglucosamination (*O*-GlcNAcylation). Levels of *O*-GlcNAcylation of Sp1 were most increased in rapamycin (100 nM)-treated cells and LY294002 (30 μM)-treated cells, approximately 350 and 200% of control. Conversely, IGF-1 (13 nM)-treated cells exhibited less Sp1 *O*-GlcNAcylation (about 60% of control). Insulin and EGF had no significant effect on Sp1 *O*-GlcNAcylation. Furthermore, total levels of phospho-serine Sp1 were higher in IGF-1-treated cells, about 140% of control, but less in rapamycin-treated cells, about 70% of control. LY294002, EGF, and insulin had no effect on Sp1 total serine phosphorylation. Finally, none of the treatments induced a significant change in total Sp1 tyrosine and threonine phosphorylation nor on total Sp1 SUMOylation (Figure 7A,B).

Effect of IGF-1, insulin, and EGF result on activation of AKT vary with time

IGF-1, insulin, and EGF had different effects on Sp1 localization and IGFBP-2 mRNA abundance (Figures 1 and 5). To explore the possibility that this was attributable to different degrees of AKT activation, we measured phospho-serine 473 AKT levels under each treatment. Figure 8A,B shows that IGF-1 (13 nM) is the most potent activator of AKT after 15 min, about 50% more potent than insulin (172 nM) and EGF (171 nM). Also, Figure 8A shows, as expected, inhibition of AKT by LY294002 (30 μM) and inhibition of p70 S6 Kinase by rapamycin (100 nM), confirming the known actions of these compounds. After 72 h, however, IGF-1-treated cells showed much more increased phospho-serine 473 AKT levels compared to insulin or EGF-treated cells (Figure 8C).

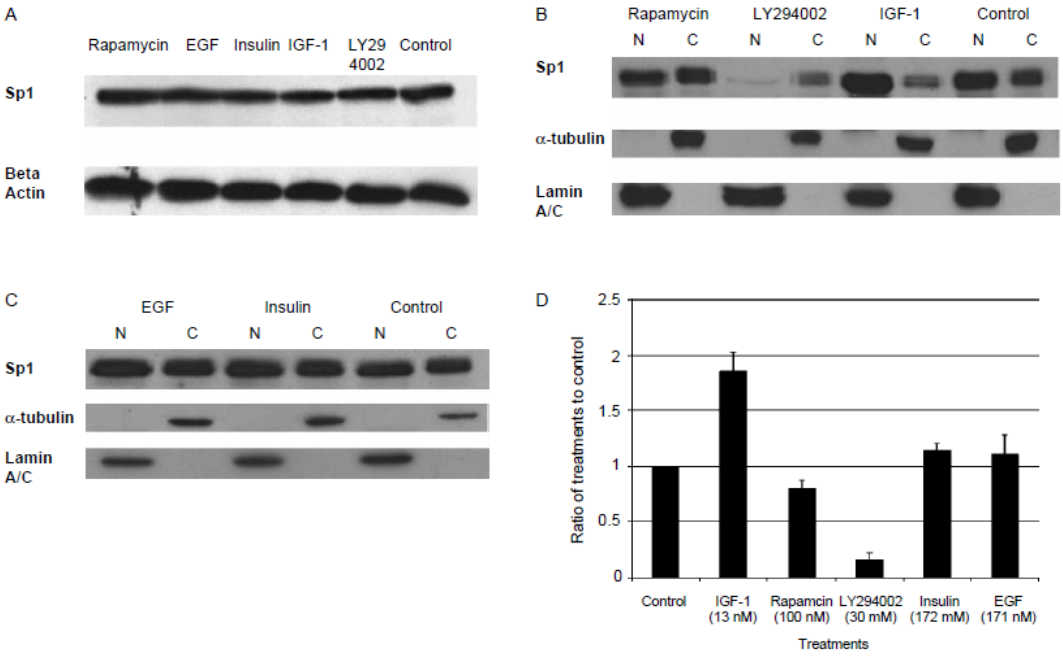


Figure 5. Effect of PI3K/AKT/mTOR pathway modulators on Sp1 protein nuclear and cytoplasmic abundance. (A) Effect on total Sp1 protein. Cells were cultured as described in previous figures and treated with appropriate growth factors (IGF-1 at 13 nM, insulin at 172 nM, EGF at 171 nM, LY294002 at 30 μM, and rapamycin at 100 nM). Total cell extracts were performed after 72 h of treatment and were immunoblotted against an anti-Sp1 antibody (PEP-2). Beta-actin is provided as a loading control. (B),(C) Effect on nuclear Sp1 abundance. Cells were fractionated after 72 h of treatment into cytoplasmic (C) and nuclear (N) extracts. α-Tubulin and lamin A/C are provided as technical controls for cell fractionation. (D) Densitometric measurements of Sp1 nuclear abundance. Sp1 nuclear abundance was quantified densitometrically (normalized to lamin A/C) and results are shown as mean ratios of treated cells to untreated control cells. Error bars indicate SEM from two independent experiments.

It has previously been shown that IGF-1 increases IGFBP-2 protein expression in an IGF-1 receptor and PI3K-dependent fashion (Martin and Baxter 2007), as inhibitors of IGF-1 receptor (AG1024) and of PI3K (LY294002) reverse the effects of IGF-1 on IGFBP-2 protein expression but inhibitors of p38 MAPK (SB203580) or p44/p42 MAPK/ERK1/2 (PD98059) do not. We confirm that this pattern is also seen for the IGFBP-2 proximal promoter-driven luciferase expression. The 200 bp sequence construct-transfected cells exhibit, as previously shown, an upregulation in luciferase expression when treated with IGF-1 (13 nM), but this effect is abrogated when cells are treated with AG1024 (10 μM) or LY294002 (30 μM). On the other hand, PD98059 (30 μM) and SB203580 (10 μM) have no effect on IGF-1-induced expression of luciferase. Thus, IGF-1 increases the 200 bp-driven luciferase expression in the same IGF1-R and PI3K-dependent fashion (Figure 9).

Discussion

Although regulation of IGFBP-2 protein levels has been associated with the PI3K/AKT/mTOR pathway, an important gap in knowledge existed with respect to the mechanism by which this regulation is achieved. We report here that the changes previously observed by others at the protein level are due mainly to regulation of IGFBP-2 mRNA by this pathway. Specifically, IGF-1 increases IGFBP-2 mRNA abundance while LY294002 and rapamycin decrease it. The observation related to rapamycin is contrary to what might have been anticipated given its well-characterized effects on mTOR and protein translation (Gingras et al. 2004). Our luciferase assays demonstrate that this pathway achieves regulation of IGFBP-2 mRNA through a regulatory region present in the first 200 bp upstream of the IGFBP-2 transcription start site. This region has been shown to bind Sp1 transcription factor in the rat. By ectopic overexpression of Sp1, we show that this transcription factor increases expression of luciferase when this 200 bp sequence is placed upstream. Additionally, we demonstrate by chromatin immunoprecipitation an increased binding of Sp1 at the IGFBP-2 proximal promoter region in IGF-1-treated cells and a decreased binding in rapamycin and LY294002-treated cells. We show that manipulation of the PI3K/AKT/mTOR pathway does not induce any change in total Sp1, but alters nuclear abundance

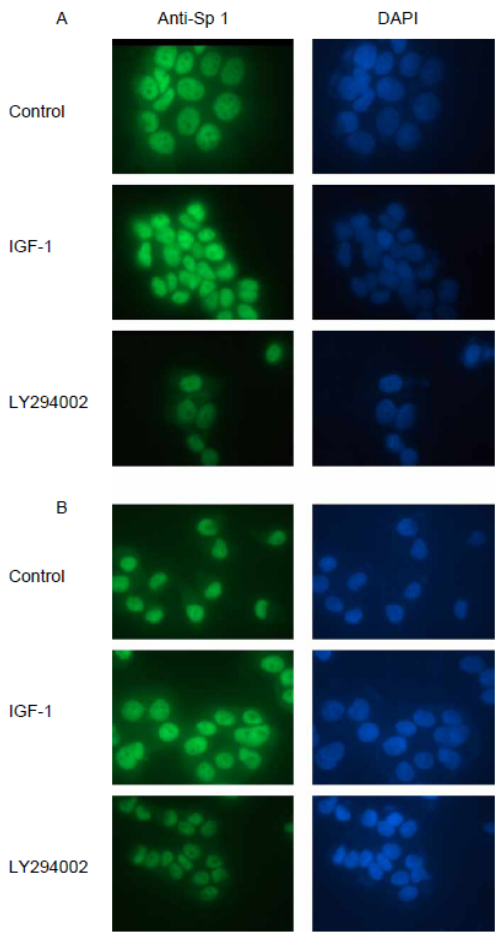


Figure 6. Immunofluorescence staining of Sp1. (A) MCF-7 cells were seeded at 5×10^4 cells per well in a Labtech eight-well microscopy slide and left to incubate for 24 h. Then, the media were changed to serum-free with the appropriate treatments [IGF-1 (13 nM) or LY294002 (30 μM)] for 72 h before immunostaining. DAPI staining indicates nuclei. (B) Same as (A) with the T47D cell line.

of the transcription factor in the same direction and at a comparable magnitude as the changes induced in IGFBP-2 protein levels. Furthermore, we demonstrate that levels of *O*-GlcNAcylation of Sp1 correlate negatively with its nuclear abundance and that total levels of phospho-serine Sp1 partially correlate with its nuclear abundance. We also demonstrate that IGF-1 induces an increase in IGFBP-2 proximal promoter-driven luciferase in a PI3K and IGF-1 receptor-dependent manner. This provides a mechanistic explanation for the observed effects of the PI3K/AKT/mTOR pathway on IGFBP-2 protein levels.

Our experiments have provided an example of an unusual effect of rapamycin, a well-known inhibitor of translation (Gingras et al. 2004), on IGFBP-2 gene transcription. We show that in MCF-7 cells, inhibition of mTOR by rapamycin leads to a decrease in nuclear Sp1 levels, but not in total Sp1 levels. Similar observations have been reported in yeast where mTOR regulates nutrient metabolism by sequestration of specific transcription factors in the cytoplasm (Beck and Hall 1999; Hardwick et al. 1999). In humans, mTOR regulation of gene transcription has only been observed with respect to ribosomal genes and rRNA. More precisely, it has been shown that the activity and cellular compartmentalization of transcription factor TIF-1A, whose involvement in RNA Pol I transcription is crucial, is modulated by mTOR. This regulation is achieved by a complex balance of kinases and phosphatases modulating two oppositely acting phosphorylation sites on TIF-1A (Mayer et al. 2004). Although mTOR does not phosphorylate TIF-1A directly, protein phosphatase 2A, a target of mTOR whose activity increases quickly after rapamycin treatment, has been shown to alter phosphorylation level and cellular localization of TIF-1A (Mayer et al. 2004). In the case of Sp1, we have shown that rapamycin treatment increases *O*-GlcNAc levels and decreases total phospho-serine levels of the transcription factor, two posttranslational modifications reported to influence Sp1 nuclear abundance (Solomon et al. 2008). The molecular mechanisms underlying these posttranslational modifications and the precise residues of Sp1 involved remain, however, unknown.

Majumdar et al. (2006) showed that insulin induced nuclear accumulation and reciprocal *O*-GlcNAcylation and phosphorylation on serine residues of Sp1 in H-411E liver cells. They reported that serine *O*-GlcNAcylation accumulates rapidly on Sp1 after treatment and declines thereafter as it is replaced by phosphorylation and as Sp1 total nuclear levels rise. Consistently, our results show that IGF-1-treated cells (which exhibited the highest nuclear Sp1 level) displayed a decrease in Sp1 *O*-GlcNAcylation and an increase in Sp1 serine phosphorylation. Rapamycin-treated cells, which displayed lower total nuclear Sp1 levels, showed increased *O*-GlcNAcylation and decreased serine phosphorylation of Sp1. Surprisingly, LY294002-treated cells, which induced the most dramatic decline in nuclear Sp1 levels, displayed increased Sp1 *O*-GlcNAcylation but no corresponding decrease in Sp1 serine phosphorylation, which suggests a different mechanism of action of LY294002 on Sp1 nuclear abundance. Contrary to Majumdar et al., we report no change in Sp1 nuclear abundance with insulin treatment, an observation which is likely due to the non-hepatic nature of the cell lines in our study. Although we report a correlation between increased nuclear Sp1 abundance and decreased Sp1

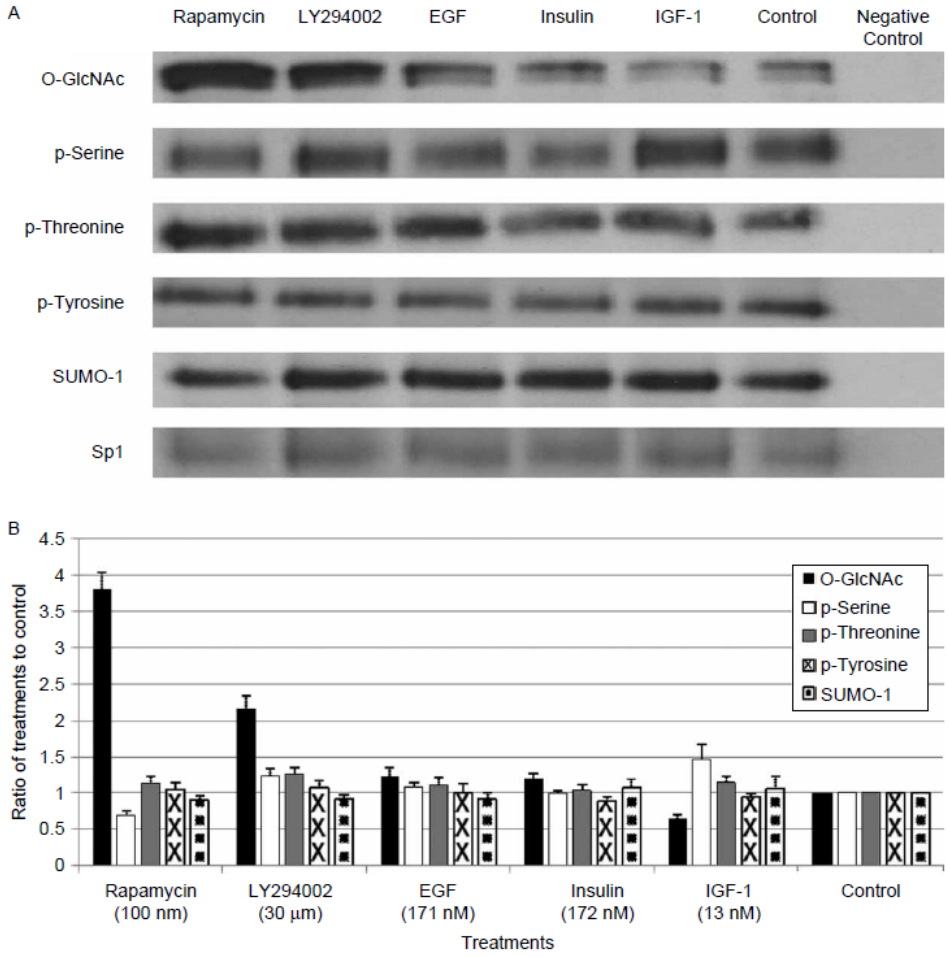


Figure 7. Effect of PI3K/AKT/mTOR pathway modulators on Sp1 posttranslational modifications. (A) Cells were cultured in 10% FBS media in 10 mm dishes until 70% confluence was reached, then treated for 72 h in serum-free media containing the appropriate compounds. Cells were then lyzed in RIPA buffer containing protease and phosphatase inhibitors, and Sp1 was immunoprecipitated and then immunoblotted with different probing antibodies against phospho-serine, phospho-tyrosine, phospho-threonine, SUMO, and *O*-GlcNAc. Then, membranes were stripped and immunoblotted with an antibody against Sp1 to provide a normalization reference (one blot is shown as example). The lane termed negative control is a lysate from control untreated cells where no anti-Sp1 antibody was added. (B) Densitometric measurements of Sp1 posttranslational modifications. Each Sp1 posttranslational modification was quantified and normalized to its corresponding total Sp1 measurement. Results are expressed as mean ratios of treatments to control and the error bars represent the SEM from two different independent experiments.

O-GlcNAcylation, and a partial correlation between an increase in total phospho-serine Sp1 and nuclear abundance, the mechanisms behind these observations remain unknown. As Sp1 contains more than 50 putative glycosylation and phosphorylation sites (Tan and Khachigian 2009), it is unclear which residues are involved in the observed changes in serine phosphorylation and *O*-GlcNacylation. Elucidation of the mechanism of Sp1 activation in the context of IGFBP-2 transcriptional modulation is the subject of ongoing work in our laboratory.

We also observed that although activation of the PI3K/AKT/mTOR pathway by IGF-1, insulin, and EGF led to comparable levels of phospho-serine 473 AKT in the short term (15 min), it was not the case in the long term (3 days). Indeed, IGF-1 was a much more potent activator of AKT after 72 h of treatment than both insulin and EGF. This difference is the probable cause of the observed discrepancies in IGFBP-2 mRNA/protein abundance and Sp1 post-translational modifications and nuclear levels between IGF-1-treated cells and insulin or EGF-treated cells.

Finally, as Sp1 is involved in the regulation of IGFBP-2 and as there is evidence that IGFBP-2 overexpression can lead to more aggressive cancer behavior by potentiating IGF-1 actions (Chen et al.

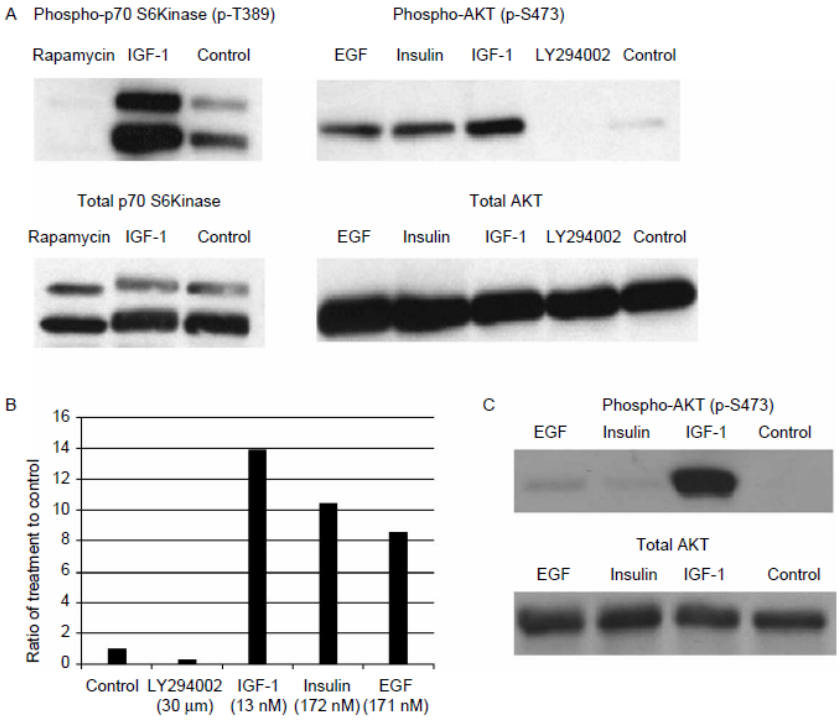


Figure 8. Signaling of PI3K/AKT/mTOR modulators. (A) Cells were seeded at 5×10^5 cells per well in six-well plates, left to incubate for 24 h, and transferred to serum-free conditions for an additional 24 h. Media were replaced with appropriate IGF-1 (13 nM), EGF (171 nM), insulin (172 nM) containing media, and cells were lyzed after 15 min. For inhibitors LY294002 (30 μM) and rapamycin (100 nM), cells were incubated in serum free medium (SFM) media for 3 h before an IGF-1 (13 nM) stimulation for 15 min. Phospho-AKT, Phospho-p70 S6Kinase, total AKT, and p70 S6Kinase were the signaling endpoints analyzed using western blotting. (B) Densitometric quantification of the effect on phospho-AKT. Phospho-AKT was densitometrically quantified (normalized to total AKT) and measurements were divided by control. (C) Same as (A) with treatments IGF-1 (13 nM), EGF (171 nM), insulin (172 nM), and 72 h cultured cells.

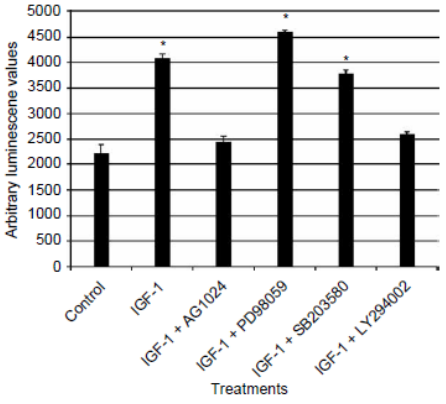


Figure 9. IGF-1 activation of the 200 bp construct. 4×10^5 cells were transfected with 2 μg of 200 bp construct. Twenty-four hours later, media were changed to serum-free control or IGF-1 (13 nM) or IGF-1 and the following AG1024 (10 μM), LY294002 (30 μM), PD98059 (30 μM), and SB203580 (10 μM). Seventy-two hours later, changes in luciferase expression were measured. Error bars are SEM and * indicates statistical significance compared to control as obtained by ANOVA ($n = 6$, p-value < 0.00001).

1994; Menouny et al. 1998; Hoeflich et al. 2000, 2001; Dunlap et al. 2007; So et al. 2008), our results motivate *in vivo* and translational research to determine whether clinical activity of Sp1 inhibitors such as Mithramycin A (which has been used in the treatment of some cancers (Kennedy and Torkelson 1995; Dutcher et al. 1997)) can be correlated with its effects on Sp1 and/or IGFBP-2. It is possible that Sp1 inhibitors will be particularly useful in cancers overexpressing IGFBP-2.

Acknowledgements

We would like to thank Dr Lilian Amrein for the important help with immunofluorescence and members of Dr Pollak's laboratory for critical discussion.

Declaration of interest: M.M. was supported by the graduate studentship from the Weekend to End Breast Cancer of the Jewish General Hospital Foundation. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Baxter R. 1993. Circulating binding proteins for the insulin-like growth factors. Trends Endocrinol Metab 4:91–96.

Beck T, Hall MN. 1999. The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. Nature 402:689–692.

Blume-Jensen P, Hunter T. 2001. Oncogenic kinase signalling. Nature 411:355–365.

Boisclair YR, Brown AL, Casola S, Rechler MM. 1993. Three clustered Sp1 sites are required for efficient transcription of the TATA-less promoter of the gene for insulin-like growth factor-binding protein-2 from the rat. J Biol Chem 268:24892–24901.

Boulle N, Logie A, Gicquel C, Perin L, Le Bouc Y. 1998. Increased levels of insulin-like growth factor II (IGF-II) and IGF-binding protein-2 are associated with malignancy in sporadic adrenocortical tumors. J Clin Endocrinol Metab 83:1713–1720.

Busund LT, Richardsen E, Busund R, Ukkonen T, Bjornsen T, Busch C, Stalsberg H. 2005. Significant expression of IGFBP2 in breast cancer compared with benign lesions. J Clin Pathol 58: 361–366.

Campbell PG, Durham SK, Hayes JD, Suwanichkul A, Powell DR. 1999. Insulin-like growth factor-binding protein-3 binds fibrinogen and fibrin. J Biol Chem 274:30215–30221.

Chan TW, Pollak M, Huynh H. 2001. Inhibition of insulin-like growth factor signaling pathways in mammary gland by pure antiestrogen ICI 182,780. Clin Cancer Res 7:2545–2554.

Chen JC, Shao ZM, Sheikh MS, Hussain A, LeRoith D, Roberts CT, Fontana JA. 1994. Insulin-like growth factor-binding protein enhancement of insulin-like growth factor-1 (IGF-1)-mediated DNA synthesis and IGF-1 binding in a human breast carcinoma cell line. J Cell Physiol 158:69–78.

Chesik D, De Keyser J, Wilczak N. 2007. Insulin-like growth factor binding protein-2 as a regulator of IGF actions in CNS: Implications in multiple sclerosis. Cytokine Growth Factor Rev 18:267–278.

Courey AJ, Tjian R. 1988. Analysis of Sp1 *in vivo* reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. Cell 55:887–898.

Drop SLS, Schuller AGP, Lindenbergh-Kortleve DJ, Groffen C, Brinkman A, Zwarthoff EC. 1992. Structural aspects of the IGFBP family. Growth Regul 2:69–79.

Dunlap SM, Celestino J, Wang H, Jiang R, Holland EC, Fuller GN, Zhang W. 2007. Insulin-like growth factor binding protein 2 promotes glioma development and progression. Proc Natl Acad Sci USA 104:11736–11741.

Dutcher JP, Coletti D, Paietta E, Wiernik PH. 1997. A pilot study of alpha-interferon and plicamycin for accelerated phase of chronic myeloid leukemia. Leuk Res 21:375–380.

Elminger MW, Bell M, Schuett BS, Langkamp M, Kutoh E, Ranke MB. 2001. Transactivation of the IGFBP-2 promoter in human tumor cell lines. Mol Cell Endocrinol 175:211–218.

Ernst CW, White ME. 1996. Hormonal regulation of IGF-binding protein-2 expression in proliferating C2C12 myoblasts. J Endocrinol 149:417–429.

Firth SM, Baxter RC. 2002. Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev 23:824–854.

Fottner C, Sattarova S, Hoffmann K, Spottl G, Weber MM. 2008. Elevated serum levels of IGF-binding protein 2 in patients with non-seminomatous germ cell cancer: Correlation with tumor markers alpha-fetoprotein and human chorionic gonadotropin. Eur J Endocrinol 159:317–327.

Frommer KW, Reichenmiller K, Schutt BS, Hoeflich A, Ranke MB, Dodt G, Elmlinger MW. 2006. IGF-independent effects of IGFBP-2 on the human breast cancer cell line Hs578T. J Mol Endocrinol 37:13–23.

Gingras AC, Raught B, Sonenberg N. 2004. mTOR signaling to translation. Curr Top Microbiol Immunol 279:169–197.

Han HJ, Kang CW, Park SH. 2006. Tissue-specific regulation of insulin-like growth factors and insulin-like growth factor binding proteins in male diabetic rats *in vivo* and *in vitro*. Clin Exp Pharmacol Physiol 33:1172–1179.

Hardwick JS, Kuruvilla FG, Tong JK, Shamji AF, Schreiber SL. 1999. Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins. Proc Natl Acad Sci USA 96:14866–14870.

Hoeflich A, Lahm H, Blum W, Kolb H, Wolf E. 1998. Insulin-like growth factor-binding protein-2 inhibits proliferation of human embryonic kidney fibroblasts and of IGF-responsive colon carcinoma cell lines. FEBS Lett 434:329–334.

Hoeflich A, Fettscher O, Lahm H, Blum WF, Kolb HJ, Engelhardt D, Wolf E, Weber MM. 2000. Overexpression of insulin-like growth factor-binding protein-2 results in increased tumorigenic potential in Y-1 adrenocortical tumor cells. Cancer Res 60:834–838.

Hoeflich A, Reisinger R, Lahm H, Kiess W, Blum WF, Kolb HJ, Weber MM, Olf E. 2001. Insulin-like growth factor-binding protein 2 in tumorigenesis: Protector or promoter? Cancer Res 61:8601–8610.

Hwa V, Oh Y, Rosenfeld RG. 1999. The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocr Rev 20:761–787.

Jones JI, Clemmons DR. 1995. Insulin-like growth factors and their binding proteins: Biological actions. Endocr Rev 16:3–34.

Kennedy BJ, Torkelson JL. 1995. Long-term follow-up of stage III testicular carcinoma treated with mithramycin (plicamycin). Med Pediatr Oncol 24:327–328.

Lalou C, Lassarre C, Binoux M. 1996. Isolation and characterization of proteolytic fragments of insulin-like growth factor-binding protein-3. Horm Res 45:156–159.

Levitt RJ, Georgescu MM, Pollak M. 2005. PTEN-induction in U251 glioma cells decreases the expression of insulin-like growth factor binding protein-2. Biochem Biophys Res Commun 336: 1056–1061.

Majumdar G, Harrington A, Hungerford J, Martinez-Hernandez A, Gerling IC, Raghow R, Solomon S. 2006. Insulin dynamically regulates calmodulin gene expression by sequential *o*-glycosylation and phosphorylation of sp1 and its subcellular compartmentalization in liver cells. J Biol Chem 281:3642–3650.

Martin JL, Baxter RC. 2007. Expression of insulin-like growth factor binding protein-2 by MCF-7 breast cancer cells is regulated through the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin pathway. Endocrinology 148: 2532–2541.

Mayer C, Zhao J, Yuan X, Grummt I. 2004. mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability. Genes Dev 18:423–434.

McDonald KL, O'Sullivan MG, Parkinson JF, Shaw JM, Payne CA, Brewer JM, Young L, Reader DJ, Wheeler HT, Cook RJ, Biggs MT, Little NS, Teo C, Stone G, Robinson BG. 2007. IQGAP1 and IGFBP2: Valuable biomarkers for determining prognosis in glioma patients. J Neuropathol Exp Neurol 66: 405–417.

Menouny M, Binoux M, Babajko S. 1998. IGFBP-2 expression in a human cell line is associated with increased IGFBP-3 proteolysis, decreased IGFBP-1 expression and increased tumorigenicity. Int J Cancer 77:874–879.

Mohan S, Baylink DJ. 2002. IGF-binding proteins are multifunctional and act via IGF-dependent and -independent mechanisms. J Endocrinol 175:19–31.

Okabe E, Kajihara J, Usami Y, Hirano K. 1999. The cleavage site specificity of human prostate specific antigen for insulin-like growth factor binding protein-3. FEBS Lett 447:87–90.

Parker A, Rees C, Clarke J, Busby WH, Jr, Clemmons DR. 1998. Binding of insulin-like growth factor (IGF)-binding protein-5 to smooth-muscle cell extracellular matrix is a major determinant of the cellular response to IGF-I. Mol Biol Cell 9:2383–2392.

Perks CM, Vernon EG, Rosendahl AH, Tonge D, Holly JM. 2007. IGF-II and IGFBP-2 differentially regulate PTEN in human breast cancer cells. Oncogene 26:5966–5972.

Richardsen E, Ukkonen T, Bjornsen T, Mortensen E, Egevad L, Busch C. 2003. Overexpression of IGBFB2 is a marker for malignant transformation in prostate epithelium. Virchows Arch 442:329–335.

So A, Levitt RJ, Eigl B, Fazil L, Muramaki M, Leung S, Cheang M, Nielsen T, Gleave M, Pollak M. 2008. Insulin-like growth factor binding protein-2 is a novel therapeutic target associated with breast cancer. Clin Cancer Res 14:6944–6954.

Solomon SS, Majumdar G, Martinez-Hernandez A, Raghow R. 2008. A critical role of Sp1 transcription factor in regulating gene expression in response to insulin and other hormones. Life Sci 83:305–312.

Sommer A, Spratt SK, Tatsuno GP, Tressel T, Lee R, Maack CA. 1993. Properties of glycosylated and non-glycosylated human recombinant IGF binding protein-3 (IGFBP-3). Growth Regul 3:46–49.

Tan NY, Khachigian LM. 2009. Sp1 phosphorylation and its regulation of gene transcription. Mol Cell Biol 29:2483–2488.

Yu J, Iwashita M, Kudo Y, Takeda Y. 1998. Phosphorylated insulin-like growth factor (IGF)-binding protein-1 (IGFBP-1) inhibits while non-phosphorylated IGFBP-1 stimulates IGF-I-induced amino acid uptake by cultured trophoblast cells. Growth Horm IGF Res 8:65–70.

Zumkeller W, Schwander J, Mitchell CD, Morrell DJ, Schofield PN, Preece MA. 1993. Insulin-like growth factor (IGF)-I, -II and IGF binding protein-2 (IGFBP-2) in the plasma of children with Wilms' tumor. Eur J Cancer 29A:1973–1977.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 19, pp. 16567–16573, May 13, 2011

Binding between Insulin-like Growth Factor 1 and Insulin-like Growth Factor-binding Protein 3 Is Not Influenced by Glucose or 2-Deoxy-D-glucose

Received for publication, December 15, 2010, and in revised form, February 23, 2011 Published, JBC Papers in Press, March 9, 2011, DOI 10.1074/jbc.M110.213033

Matei Mireuta[‡], **Mark A. Hancock**[§], **and Michael Pollak**[‡1]

From the [‡]*Segal Cancer Centre and Lady Davis Institute of the Jewish General Hospital, Montreal, Quebec H3T 1E2 and the* [§]*Sheldon Biotechnology Centre, McGill University, Montreal, Quebec H3A 2B4, Canada*

A recent report (Zhong, D., Xiong, L., Liu, T., Liu, X., Liu, X., Chen, J., Sun, S. Y., Khuri, F. R., Zong, Y., Zhou, Q., and Zhou, W. (2009) *J. Biol. Chem.* 284, 23225–23233) details that 2-deoxy-D-glucose (2-DG), a well known inhibitor of glycolysis and a candidate antineoplastic agent, also induces insulin-like growth factor 1 receptor (IGF-1R) signaling through the inhibition of insulin-like growth factor 1-insulin-like growth factor-binding protein 3 (IGF-1-IGFBP-3) complex formation. Zhong *et al.* hypothesized that disrupted IGF-1/IGFBP-3 binding by 2-DG led to increased free IGF-1 concentrations and, consequently, activation of IGF-1R downstream pathways. Because their report suggests unprecedented off-target effects of 2-DG, this has profound implications for the fields of metabolism and oncology. Using ELISA, surface plasmon resonance, and novel "intensity-fading" mass spectrometry, we now provide a detailed characterization of complex formation between IGF-1 and IGFBP-3. All three of these independent methods demonstrated that there was no effect of glucose or 2-DG on the interaction between IGF-1 and IGFBP-3. Furthermore, we show examples of 2-DG exposure associated with reduced rather than increased IGF-1R and AKT activation, providing further evidence against a 2-DG increase in IGF-1R activation by IGF-1-IGFBP-3 complex disruption.

Insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) are peptide hormones similar in molecular structure to insulin, and they regulate a variety of cellular activities, including metabolism, proliferation, and growth. Both IGFs[2] bind to the IGF-1 receptor (IGF-1R) at the cell membrane and initiate a signaling cascade that results in the activation of the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin pathway (1–5). The IGF-2 receptor, which binds to IGF-2, lacks an intracellular signaling domain and is therefore considered to only act as a sink for excess IGF-2 (6).

IGF actions are tightly modulated by a family of proteins called insulin-like growth factor-binding proteins (IGFBPs), of which IGFBP-1–6 have been characterized. Most IGFBPs in the blood originate from the liver, but they are also expressed in many other tissues. IGFBP-3 and -5 are the most abundant IGFBPs in the circulation. They form a ternary complex with IGFs and a third protein termed acid-labile subunit (7–9). Insulin-like growth factor-binding proteins are known to modulate actions of IGFs both *in vitro* and *in vivo* (7). Because only free IGFs are considered ligands for the IGF-1R, significant research efforts have focused on small molecules capable of interfering with IGF-IGFBP binding.

A recent report showed that 2-deoxy-D-glucose (2-DG), a close derivative of glucose, promoted dissociation of IGF-1 from IGFBP-3 and consequently contributed to elevated phosphoserine 473 AKT levels in a variety of cancer cell lines (10). 2-DG has been proposed as a potential therapeutic agent in the treatment of cancer because it interferes with glycolysis (11). Entering the cell through glucose transporters, 2-DG inhibits enzymes of the glycolytic pathway both competitively (phosphoglucose isomerase) (12) and noncompetitively (hexokinase) (13). Because tumor cells depend more heavily on glycolysis compared with normal cells, 2-DG is under investigation for cancer treatment (14).

The recent study by Zhong *et al.* (10) showed that 2-DG, apart from its classic activities as an inhibitor of glycolysis, can also increase IGF-1R signaling by disrupting IGF-1/IGFBP-3 binding. As increased IGF-1R signaling is associated with greater proliferation of tumor cells, this report has shed doubt on the efficacy of 2-DG as a cancer therapeutic. To examine this issue further, this study characterizes the effect of glucose and 2-DG on binding between IGF-1 and IGFBP-3. Using ELISA, surface plasmon resonance (SPR), and novel "intensity-fading" mass spectrometry (MS), we report that glucose and 2-DG have no effect on IGF-1-IGFBP-3 complex formation.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant IGF-1 was purchased from Feldan Biosciences (Montreal, Canada), and human recombinant IGFBP-3 was obtained from Insmed Inc. (Glen Allen, VA). Glucose, 2-DG, and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma. MCF-7, T47D, and HeLa cell lines were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and cultured in standard DMEM m supplemented with 10% fetal bovine serum (FBS) and 20 μg/ml gentamycin. Phosphate buffered saline (PBS; 10 mM Sodium Phosphate, 2.7 mM KCl, 137 mM NaCl) and Hepes buffered saline (HBS-EP; 10 mM Hepes pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) Tween-20) were prepared using analytical grade chemicals.

[1] To whom correspondence should be addressed: 3755 Cote-Ste Catherine, Montreal, Quebec H3T 1E2, Canada. Tel.: 514-340-8222 (Ext. 4139); Fax: 514-340-8222; E-mail: michael.pollak@mcgill.ca.

[2] The abbreviations used are: IGF, insulin-like growth factor; IGF-1R, IGF-1 receptor; 2-DG, 2-deoxy-D-glucose; RU, resonance unit; SPR, surface plasmon resonance; IGFBP, insulin-like growth factor-binding protein.

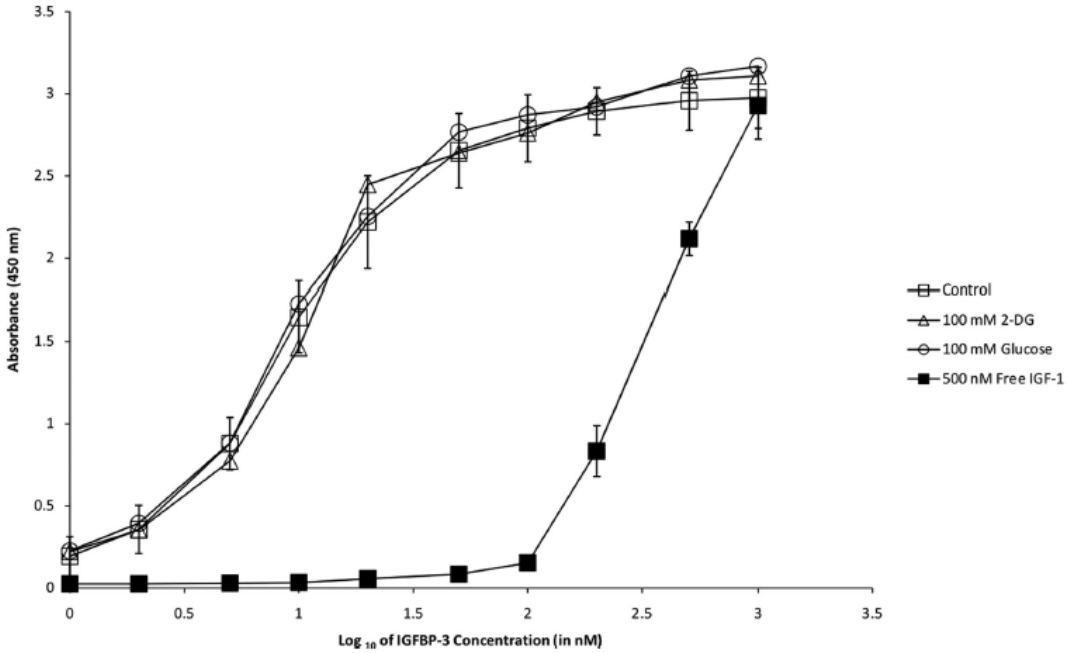


FIGURE 1. **ELISA to monitor binding of IGFBP3 (0–1000 nM in HBS-EP) to immobilized IGF-1 in the absence (*open squares*) or presence of 100 mM 2-DG (*open triangles*), 100 mM glucose (*open circles*), and 500 nM IGF-1 (*closed squares*).** *Error bars* (standard deviation of triplicate measurements) are only depicted for the "control" and "500 nM IGF-1" series for simplicity.

ELISA—Microtiter plates (Costar, Lowell, MA) were coated with either human recombinant IGF-1 (5 μg/ml) or human recombinant IGFBP-3 (2 μg/ml) in PBS overnight at room temperature. Plates were washed three times with sample buffer (PBS or HBS-EP; 100 μl/well) and then blocked for 3 h at room temperature using 5% (w/v) casein (in sample buffer; 300 μl/well). Plates were washed again in a similar manner before 30-min incubations with IGFBP-3 or 2.5-h incubations with IGF-1 at room temperature. After washing, primary antibodies (anti-IGFBP-3 or anti IGF-1; Santa Cruz Biotechnology, Santa Cruz, CA) were added to each well (100 μl of 1:50 dilution in sample buffer containing 5% (w/v) casein) for 1 h at room temperature. After washing, secondary antibody (Santa Cruz Biotechnology) was added to each well in the similar manner. After washing, the color developing solution (R&D Systems, Minneapolis, MN) was added to the wells, and absorbance readings were monitored at 450 nm.

SPR—Label-free, real time binding between IGF-1 (7.6 kDa) and IGFBP-3 (29 kDa) was examined using a Biacore 3000 system (GE Healthcare) at 25 °C with filtered (0.2 μm) and degassed HBS-EP running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) Tween 20). IGFBP-3 (9 μg/ml in 10 mM sodium acetate, pH 5.5) was immobilized to CM4 sensor chips using the Biacore amine coupling kit (~1000 RU final); corresponding reference surfaces were prepared in the absence of IGFBP-3. IGF-1 (0–25 nM) (or BSA as negative control) was injected over reference and IGFBP-3-immobilized surfaces, in the absence or presence of competitor (25–100 mM glucose or 2-DG), using variable flow rates (10–75 μl/min) and contact times (3–5 min association, 5–10 min dissociation). Between sample injections, sensor chip surfaces were regenerated using Pierce gentle elution buffer (Thermo Scientific) containing 0.1% (v/v) Empigen (Affymetrix-Anatrace).

All SPR data were double-referenced (15) and are representative of duplicate injections acquired from two independent trials. For each titration series, a buffer blank (± glucose or 2-DG) was injected first, the highest IGF-1 concentration second, and serial dilutions then followed (from the lowest to the highest concentration repeated). Comparing binding responses between the highest IGF-1 injections verified consistent IGFBP-3-immobilized surface activity throughout each assay. Apparent equilibrium dissociation constants (K_D), as well as individual association (k_a) and dissociation (k_d) rate constants, were determined by global fitting of the data to a "1:1 kinetic" model with or without mass transport (BIAevaluation version 4.1 software). The kinetic estimates represent fits to the experimental data where χ^2 values were <1.5.

MS—Eppendorf tubes containing IGF-1 (13 μM) or IGF-1 plus IGFBP-3 (13 or 26 μM) samples were incubated for 3 h on a rocking platform at room temperature. A normal phase (NP-20) protein chip (Bio-Rad) was washed three times with 5 μl of HPLC-grade water before the addition of 5 μl of sample per spot and air drying. Subsequently, each spot was washed three times with 5 μl of a low stringency buffer (Bio-Rad) and then left to air dry. 5 mg of α-cyano-4-hydroxycinnamic acid (Bio-Rad) was dissolved in 200 μl of solution A (50% (v/v) HPLC-grade water, 49.5% (v/v) acetonitrile, and 0.5% (v/v) trifluoroacetic acid); the mixture was vortexed for 5 min and then

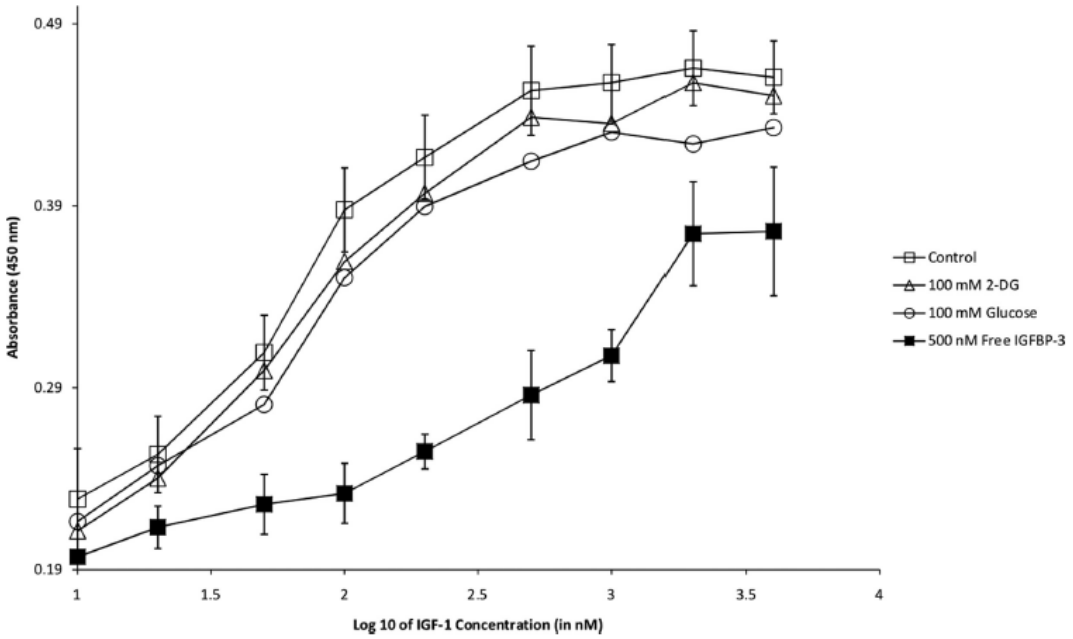


FIGURE 2. **Reverse ELISA to monitor binding of IGF-1 (0–5000 nM in HBS-EP) to immobilized IGFBP-3 in the absence (*open squares*) or presence of 100 mM 2-DG (*open triangles*), 100 mM glucose (*open circles*), and 500 nM IGFBP-3 (*closed squares*).** *Error bars* (standard deviation of triplicate measurements) are only depicted for the control and 500 nM IGFBP-3 series for simplicity.

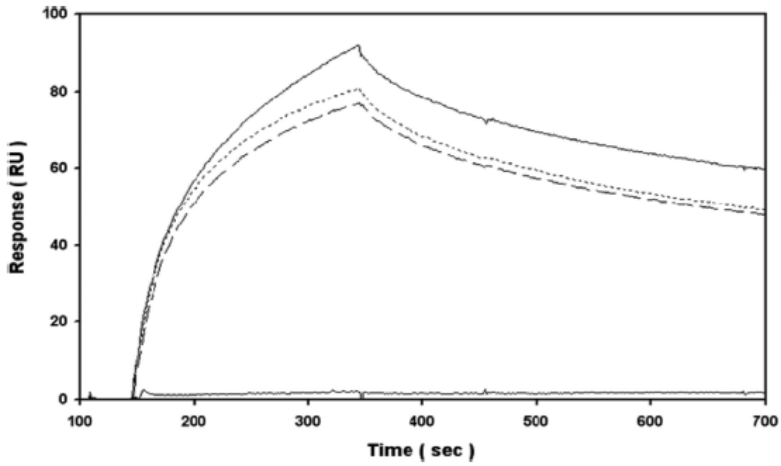


FIGURE 3. **SPR analysis to monitor specificity of IGF-1 (25 nM in HBS-EP) binding to IGFBP-3 (1000 RU amine-coupled) in the absence (*upper solid line*) or presence of 100 mM 2-DG (*dashed line*) and 100 mM glucose (*dotted line*) at 75 μl/min.** As a negative binding control, no significant response was observed with 25 nM BSA (*bottom solid line*). Double-referenced data are representative of duplicate injections acquired from two independent trials.

centrifuged for 10 min before the supernatant was collected and further diluted with 200 μl of solution A. The diluted supernatant was then added to each spot (1 μl per spot) and left to air dry; this was repeated twice to create two layers. The chip was then analyzed using a Ciphergen Protein Chip Series 4000 instrument (Ciphergen Biosystems, Fremont, CA).

Western Blots—Total cell lysates were obtained using RIPA buffer as described earlier (16). 50 μg of total protein were loaded per lane, and membranes were immunoblotted with the following antibodies: phosphoserine 473 AKT, AKT, phosphotyrosine 1135/1136 IGF-1R β chain, IGF-1R β chain, and β-actin (Cell Signaling, Danvers, MA).

RESULTS

Qualitative Binding of IGF-1 to IGFBP-3—Initially, ELISAs were performed in which IGF-1 was immobilized in the micro-

titer wells (Fig. 1). In an HBS-EP buffer system, saturable, dose-dependent binding of IGFBP-3 was detected and yielded sigmoidal curves (log scale) as expected for typical protein interactions. Binding specificity was evidenced by decreased colorimetric responses in the presence of free IGF-1 (Fig. 1), as well as high sodium chloride and high Tween 20 detergent concentrations (data not shown). In the presence of excess glucose or 2-DG (25–100 mM), however, the binding of IGFBP-3 to immobilized IGF-1 was unaltered in all cases. As negative controls, microtiter wells lacking immobilized protein or primary/secondary antibodies failed to generate any colorimetric response (data not shown). In the reversed orientation, ELISAs were also performed in which IGFBP-3 was immobilized (Fig. 2). Saturable, dose-dependent binding of IGF-1 was detected, and similar to above, the interaction could be competed with free IGFBP-3 but was unaltered by glucose or 2-DG. ELISAs were performed in other buffer settings (PBS, Krebs-Ringer) with identical conclusions (data not shown).

Quantitative Binding of IGF-1 to IGFBP-3—To validate the ELISA data, binding between IGF-1 and IGFBP-3 was then examined using label-free, real time SPR and the identical HBS-EP buffer system. A single low nanomolar concentration of IGF-1 specifically bound to immobilized IGFBP-3 under high flow rate conditions (Fig. 3), whereas an equimolar concentration of BSA failed to interact (*i.e.* negative binding control). The overall amounts of IGF-1 bound, and the individual association and dissociation kinetics were not significantly altered in the presence of excess glucose or 2-DG (25–100 mM). Expanding upon the fixed concentration specificity tests, IGF-1 was then titrated in the absence and presence of excess 2-DG (Fig. 4). Visually, the overall dose dependence and individual kinetics of IGF-1 binding to immobilized IGFBP-3 were not significantly altered in the presence of excess glucose or 2-DG. Likewise, global fitting of each titration series to a 1:1 kinetic model indicated that there was no significant difference in the subnanomolar affinity for this interaction over replicate trials (Table 1). In the absence or presence of an additional "k_t" parameter, we also confirmed that the curve fitting was not significantly altered by mass transport limitations (*i.e.* fitted k_t coefficient was $\sim 10^8$ RU M^{-1} s^{-1} as expected).

Complex Formation between IGF-1 and IGFBP3—To validate the ELISA and SPR data, we also used MS to demonstrate that 2-DG and glucose have no effect on IGF-1/IGFBP-3 binding. In recent years, surface-enhanced laser desorption/ionization and matrix-enhanced laser desorption/ionization (MALDI)-time of flight (TOF) MS have become powerful tools for the investigation of noncovalent protein complexes (17). Intensity fading experiments are relatively novel techniques used to directly visualize protein-protein binding without the use of cross-linkers (18). In these experiments, a fixed concentration of protein A (ideally between 5 and 16 kDa) is incubated with different concentrations of its binding partner B; as the concentration of protein B is increased, the intensity of the peak corresponding to protein A slowly diminishes or "fades" (19).

In our MS experiments, we incubated IGF-1 at 13 μM alone, with an equimolar concentration of IGFBP-3 (13 μM), or with excess IGFBP-3 (26 μM) in PBS. Fig. 5*A* shows that the intensity

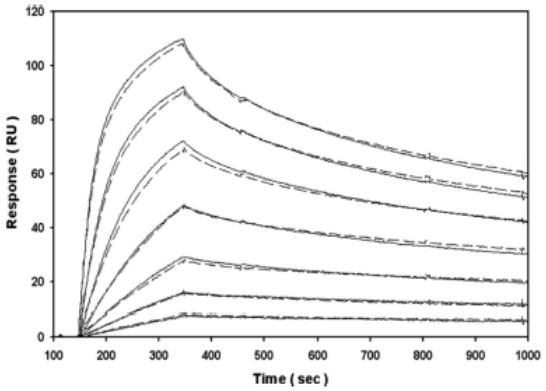


FIGURE 4. **SPR analysis to monitor real time kinetics of IGF-1 (0–25 nM in HBS-EP; 2-fold dilution series) binding to IGFBP-3 (1000 RU amine-coupled) in the absence (*solid lines*) or presence of 100 mM 2-DG (*dashed lines*) at 75 μl/min (3. 5 min association + 10 min dissociation).** Double-referenced data are representative of duplicate injections acquired from two independent trials.

TABLE 1

Apparent kinetics of IGF-1 binding to immobilized IGFBP-3 in the absence and presence of 2-DG, as assessed by SPR

Estimates (means ± S.E.; $n = 4$) represent global analysis of titration series to a 1:1 kinetic model where goodness-of-fit (χ^2) values were <1.5; association rate (k_a), dissociation rate (k_d), and equilibrium dissociation ($K_D = k_d/k_a$) constants were not significantly altered in the presence of an additional mass transport ($k_t \sim 10^8$ RU M^{-1} s^{-1}) fitting parameter.

100 mM 2-DG	$k_a \times 10^6$	$k_d \times 10^{-4}$	K_D
	M^{-1} s^{-1}	s^{-1}	pM
−	1.40 ± 0.01	8.40 ± 0.04	604 ± 5
+	1.33 ± 0.01	7.93 ± 0.05	599 ± 5

of IGF-1 (7600 Da) fades with increasing concentrations of IGFBP-3 (29,000 Da), decreasing from 1000 intensity units to under 50 at the highest IGFBP-3 concentration. Epidermal growth factor (EGF) (9 μM) was included as an internal non-binding control, and its intensity (6400 Da) remained unaltered across all IGFBP-3 concentrations. As commonly encountered in intensity fading experiments (18, 19), detection of the resultant IGF-1-IGFBP-3 complex (36,000 Da) was not proportional to theoretical expectations. The same experiments were performed in PBS containing 100 mM 2-DG (Fig. 5*B*) or 100 mM glucose (data not shown), and similar spectral outcomes were observed. Because neither glucose nor 2-DG was able to reverse the intensity fading of the IGF-1 peak, this assay indicates that they do not influence IGF-1/IGFBP-3 binding.

Effects of 2-DG on AKT and IGF-1R—Furthermore, we investigated the effect of 2-DG on IGF-1R and AKT activation in two breast cancer cell lines (MCF-7 and T47D) and one cervical cancer cell line (HeLa). We observed that addition of 2-DG at 25 mM, a concentration previously reported to enhance AKT phosphorylation in these cell lines (20), resulted in a consistent decrease in IGF-1R activation. As shown in Fig. 6, exposure to 2-DG actually reduced IGF-1R β chain tyrosine 1135/1136 phosphorylation in both basal and IGF-1 (130 nM)-stimulated contexts and across all three cell lines. We also observed that AKT activation is not universally induced by 2-DG. Fig. 6 shows that phosphoserine 473 AKT is increased in T47D cells and

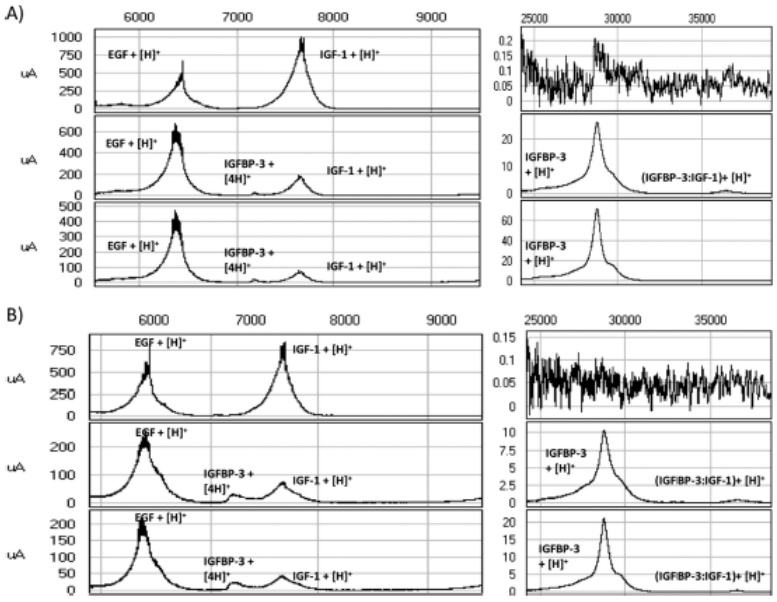


FIGURE 5. **MS analysis to monitor binding between IGF-1 (~7600 Da) and IGFBP-3 (~29,000 Da) in the absence or presence of 100 mM 2-DG (and internal EGF nonbinding control, ~6400 Da).** *A, upper boxes* represent 13 nM IGF-1 alone; *middle boxes* represent 13 nM IGF-1 with 13 nM IGFBP-3, and *lower boxes* represent 13 nM IGF-1 with 26 nM IGFBP-3. *B,* identical intensity fading experiments performed in the presence of 100 mM 2-DG. The data presented are representative of three independent experiments. *y* axes are representative of signal response (microampere (*uA*)), and *x* axes are representative of molecular mass (Da).

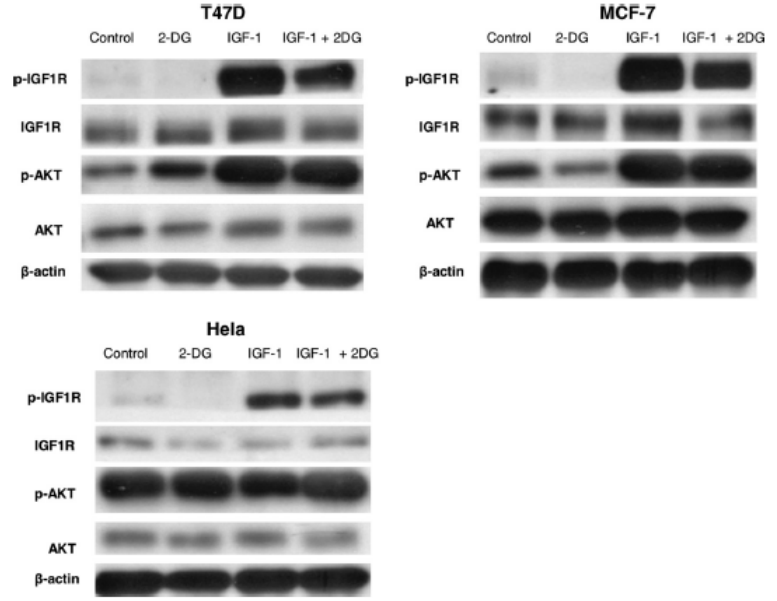


FIGURE 6. **Western blot assay of IGF-1R signaling.** MCF-7, T47D, and HeLa cell lines were plated at 10^6 cells/well in 6-well plates in DMEM containing 10% FBS. The following day, cells were treated with 10% FBS DMEM containing 2-DG (25 mM), IGF-1 (130 nM), or both. Four hours later, cells were harvested, and levels of signaling proteins were assayed by Western blot.

decreased in MCF-7 cells in basal 10% FBS conditions. In IGF-1 (130 nM)-stimulated conditions, 2-DG does not appear to have a considerable effect on AKT activation in either MCF-7 or T47D cells. In HeLa cells, AKT phosphorylation is basally high and is not substantially altered in the presence of IGF-1 or 2-DG. These results indicate that 2-DG-induced AKT

activation is not universal and not IGF-1R-dependent, as implied by Zhong *et al.* (20). Moreover, 2-DG-induced activation of 5′-adenosine monophosphate-activated protein kinase was consistently high across all cell lines (data not shown), which is in agreement with previously published results by Zhong *et al.* (20) suggesting that 2-DG-induced changes in AKT activation are 5′-adenosine monophosphate-activated protein kinase-independent.

DISCUSSION

Our main objective was to investigate the effects of 2-DG and glucose on IGF-1/IGFBP-3 binding, as a follow-up to a recent publication by Zhong *et al.* (10). They showed that a concentration of 25 mM 2-DG was sufficient to disrupt more than 60% of IGF-1-IGFBP-3 complexes, a finding that, if confirmed, would have considerable impact both on IGF and cancer research. The reported finding that 2-DG exposure is associated with higher free IGF-1 concentrations challenges its utility as an antineoplastic agent and even its utility as an experimental strategy to selectively inhibit glycolysis without affecting other aspects of cellular physiology. It became important to determine whether its relatively well characterized inhibition of glycolysis would predominate over this newly described stimulation in IGF signaling.

We sought to extend the findings of Zhong *et al.* (10) by identifying changes in affinity constants and by evaluating the potential effects of glucose, obviously structurally related to 2-DG, on IGF-1-IGFBP-3 complex formation. Using ELISA, we have shown that IGF-1/IGFBP-3 binding is unaltered by either 2-DG or glucose at various concentrations both within and exceeding physiological range. The assay was performed both in different orientations and in different buffer systems with identical conclusions.

To date, several groups have used SPR to examine the interaction between IGF-1 and its binding proteins (21, 22). Similar to the assay design detailed in previous reports, our SPR results are based upon IGF-1 injected over immobilized IGFBP-3 at a high flow rate (75 μl/min) and low signal range (<150 RU) to minimize any potential mass transport effects. Global fitting of the titration series to a 1:1 kinetic model with or without mass transport effects yielded similar outcomes. Our kinetic estimates were biologically relevant (*e.g.* $k_a \sim 10^3$–10^7 M^{-1} s^{-1} and $k_d \sim 10^{-1}$–10^{-6} s^{-1} for typical protein interactions) and correlated well with values reported previously (21, 22) (*i.e.* overall affinity between IGF-1 and IGFBP-3 is subnanomolar). Ultimately, the presence of excess glucose or 2-DG failed to significantly alter the binding interaction between IGF-1 and immobilized IGFBP-3 in our SPR assay.

Using mass spectrometry, we demonstrated for the first time that a newer intensity fading approach (19) is appropriate for the study of IGF-IGFBP complexes. Although the resultant signal for the complexes did not match the anticipated theoretical predictions, this issue has been encountered by others (18, 19) and may be the result of higher ionization energies required for complexes compared with the individual protein and its binding partner alone. Nevertheless, our intensity fading experiments clearly show that the signal corresponding to IGF-1 was decreased in a dose-dependent manner upon IGFBP-3 addition and that 2-DG or glucose had no effect compared with the control MS spectra.

Upon examination of 2-DG-induced changes in IGF-1R signaling in MCF-7, T47D, and HeLa cell lines, we were unable to confirm the previously published finding that exposure to 2-DG induces IGF-1R activation (10). In fact, we observed that 2-DG treatment reduces phosphorylation of IGF-1R in all three cell lines tested. This finding provides further evidence against the hypothesis that 2-DG disrupts IGF-1-IGFBP-3 complex formation, which leads to activation of the IGF-1R. Moreover, the fact that phosphoserine 473 AKT levels changed in a cell-specific manner and were uncorrelated with phospho-IGF-1R levels supports the hypothesis that 2-DG-induced changes in AKT activation are independent of IGF-1R in these cell lines.

In conclusion, we have utilized three unique experimental strategies to demonstrate that 2-DG does not alter the binding interaction between IGF-1 and IGFBP-3. Although our direct measures of IGF-1-IGFBP-3 complex formation contrast with the experimental findings reported by Zhong *et al.* (10), we suspect that the commercial assay (which was designed to measure free IGF-1 in serum samples) utilized in their study may have been limited by its ability to provide only an indirect measure of IGF-1-IGFBP-3 complex formation. The mechanism responsible for the 2-DG-induced increase in IGF-1R signaling observed by Zhong *et al.* (10, 20) in some cell lines remains unknown. However, our results using several independent methods do not support the hypothesis of a universal mechanism involving IGF-1-IGFBP-3 complex disruption but rather suggest a mechanism involving cell-specific intracellular signaling differences.

Acknowledgments—We thank the Genome Quebec and Lady Davis Institute Clinical Proteomics Facility for their assistance with mass spectrometry experiments. Sheldon Biotechnology Centre is supported by a research resource grant from the Canadian Institutes of Health Research.

REFERENCES

1. Pollak, M. (2008) *Nat. Rev. Cancer* **8,** 915–928
2. Laviola, L., Natalicchio, A., and Giorgino, F. (2007) *Curr. Pharm. Des.* **13,** 663–669
3. Collett-Solberg, P. F., and Cohen, P. (2000) *Endocrine* **12,** 121–136
4. Vincent, A. M., and Feldman, E. L. (2002) *Growth Horm. IGF Res.* **12,** 193–197
5. LeRoith, D., and Roberts, C. T., Jr. (2003) *Cancer Lett.* **195,** 127–137
6. Ghosh, P., Dahms, N. M., and Kornfeld, S. (2003) *Nat. Rev. Mol. Cell Biol.* **4,** 202–212
7. Firth, S. M., and Baxter, R. C. (2002) *Endocr. Rev.* **23,** 824–854
8. Hwa, V., Oh, Y., and Rosenfeld, R. G. (1999) *Endocr. Rev.* **20,** 761–787
9. Jones, J. I., and Clemmons, D. R. (1995) *Endocr. Rev.* **16,** 3–34
10. Zhong, D., Xiong, L., Liu, T., Liu, X., Liu, X., Chen, J., Sun, S. Y., Khuri, F. R., Zong, Y., Zhou, Q., and Zhou, W. (2009) *J. Biol. Chem.* **284,** 23225–23233
11. Wick, A. N., Drury, D. R., and Morita, T. N. (1955) *Proc. Soc. Exp. Biol. Med.* **89,** 579–582
12. Wick, A. N., Drury, D. R., Nakada, H. I., and Wolfe, J. B. (1957) *J. Biol. Chem.* **224,** 963–969
13. Chen, W., and Guéron, M. (1992) *Biochimie* **74,** 867–873
14. Singh, D., Banerji, A. K., Dwarakanath, B. S., Tripathi, R. P., Gupta, J. P., Mathew, T. L., Ravindranath, T., and Jain, V. (2005) *Strahlenther Onkol* **181,** 507–514

15. Myszka, D. G. (1999) *J. Mol. Recognit.* **12,** 279–284
16. Levitt, R. J., Georgescu, M. M., and Pollak, M. (2005) *Biochem. Biophys. Res. Commun.* **336,** 1056–1061
17. Bolbach, G. (2005) *Curr. Pharm. Des.* **11,** 2535–2557
18. Yanes, O., Nazabal, A., Wenzel, R., Zenobi, R., and Aviles, F. X. (2006) *J. Proteome Res.* **5,** 2711–2719
19. Yanes, O., Villanueva, J., Querol, E., and Aviles, F. X. (2007) *Nat. Protoc.* **2,** 119–130
20. Zhong, D., Liu, X., Schafer-Hales, K., Marcus, A. I., Khuri, F. R., Sun, S. Y., and Zhou, W. (2008) *Mol. Cancer Ther.* **7,** 809–817
21. Beattie, J., Phillips, K., Shand, J. H., Szymanowska, M., Flint, D. J., and Allan, G. J. (2008) *Mol. Cell. Biochem.* **307,** 221–236
22. Vorwerk, P., Hohmann, B., Oh, Y., Rosenfeld, R. G., and Shymko, R. M. (2002) *Endocrinology* **143,** 1677–1685