THE NUCLEAR ACTIONS OF IGFBP-3

By

WAN-JUNG LIN

A thesis submitted in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCES

WASHINGTON STATE UNIVERSITY
Department of Animal Sciences

AUGUST 2006

To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of WAN-JUNG LIN find it satisfactory and recommend that it be accepted.

_____
Chair

_____

_____

ACKNOWLEDGEMENT

In the two years work in Animal Sciences WSU, I have been supported and accompanied by so many great people. First, I would like to thank my advisor and big brother Dan (Dr. Rodgers) who is not only a great scientist, but also a most kind person. Under his scientific supervision and enthusiasm for helping students, I learned a great deal more than I expected. In addition, I was grateful to have had worked with Momo (Dr. Oufattole), for his experienced help in the laboratory, his willingness to engage in discussions, and his encouragement for both research and life. Furthermore, I would like to thank all the members and friends of the Rodgers' lab who sweetened up my lab work every day and were always available for any questions and help. I am also grateful to have those friends who always give me mental support either in the US (Arriba) or Taiwan.

Moreover, I am thankful for the assistance and support from my committee members Dr. Rodgers, Dr. Johnson and Dr. McLean, throughout my masters thesis program.

Knowing these people is a most precious experiences, even though they couldn't realize how much I learned from them and how important they are.

Last, but not least, I would like to thank my mother, father, brother Kenny and sister Phoebe who always offered support and love.

NUCLEAR ACTIONS OF IGFBP-3

Abstract

by Wan-Jung Lin, M.S.
Washington State University
August 2006

Chair: Buel Dan Rodgers

IGF-binding protein (IGFBP)-3 has intrinsic antiproliferative and proapoptotic functions that are independent of IGF binding and may involve nuclear localization. We determined that exogenous IGFBP-3 rapidly translocates to myoblast nuclei and that a 22-residue peptide containing the metal binding domain (MBD) and nuclear localization sequence (NLS) can similarly direct chimeric GFP into myoblast nuclei. Furthermore, a non-IGF-binding IGFBP-3 mutant inhibited myoblast proliferation without stimulating apoptosis. These results suggest that IGFBP-3 inhibits muscle cell growth in an IGF-independent manner that may be influenced by its rapid nuclear localization. We therefore identified IGFBP-3 interacting proteins by screening a rat L6 myoblast cDNA library using the yeast two-hybrid assay and two N-terminal deletion mutants as bait: BP3/231 (231 residues, L61 to K291) and BP3/ 111 (K181 to K291). Proteins previously known to interact with IGFBP-3 as well as several novel proteins were identified, including RNA polymerase II binding subunit 3 (Rpb3). The domain necessary for Rpb3 binding was subsequently identified using different IGFBP-3 deletion mutants and was localized to the MBD/NLS epitope. Rpb3/IGFBP-3 binding was confirmed by

coimmunoprecipitation assays with specific antisera, whereas a NLS mutant IGFBP-3 did not associate with Rpb3, suggesting that a functional NLS is required. Rpb3 facilitates recruitment of the polymerase complex to specific transcription factors and is necessary for the transactivation of many genes. Its association of Rpb3 with IGFBP-3 provides a functional role for IGFBP-3 in the direct modulation of gene transcription.

TABLE OF CONTENTS

Page

FIGURES

TABLES

CHAPTER ONE

INTRODUCTION TO THE IGF-INDEPENDENT ROLES OF INSULIN-LIKE GROWTH FACTOR BINDING PREOTEIN (IGFBP)-3

The insulin-like growth factor (IGF) system plays a critical role in modulating cell growth and the development of the central nervous system, skeletal muscle, reproductive organs and other tissues. It includes both IGF-I and II, a single trans-membrane tyrosine-kinase receptor and six high affinity IGF binding proteins (IGFBP-1 to -6) (LeRoith & Roberts 2003, Duan & Xu 2005). Of the two factors, IGF-I is better characterized and is mainly synthesized in the liver under the primary control of growth hormone (GH). Indeed, most of GH's actions are mediated by stimulating IGF-I synthesis in hepatocytes (Grimberg & Cohen 2000) or its secretion locally, which thereby controls the growth of somatic tissues, including skeletal muscle, by paracrine and autocrine mechanisms (Adams & Gregory 2002).

The biological actions of both IGFs are transduced intracellularly by the type I IGF receptor (IGF-IR). Ligand binding to the two α subunits induces conformational changes in the intracellular β subunits, which in turn autophosphorylates (LeRoith & Roberts 2003). Activation of the β subunit tyrosine kinase domains stimulates two principle down stream signaling cascades; the mitogen-activated protein (MAP) kinase pathway (LeRoith & Roberts 2003) and the phosphoinositol-3 kinase (PI3-K) pathway (Yamamoto *et al.* 1992). Phosphorylation of Shc (SH2 containing proto-oncogene) initiates the MAP Kinase pathway and recruits other factors including the growth factor

receptor-bound 2(Grb2)/Sos complex, which subsequently activates Ras (a GTP-binding protein kinase) and the different MAPK pathways (LeRoith *et al.* 1995). This signaling cascade induces cell proliferation and differentiation by controlling the duration of MAPK activities and is largely responsible for the IGFs mitogenic actions in both normal and transformed cells (Marshall 1995). Activation of the PI3-K pathways begins with the phosphorylation of the insulin receptor substrates (IRS-1 to -4) which then recruit SH2 domain containing proteins including the p85 subunits of PI3-K (Backer *et al.* 1992). This in turn leads to the activation of Akt and stimulates cell survival by inhibiting apoptotic signals. IRS-1 also interacts with other proteins such as Syp (phosphotyrosine phosphatase), which has been shown to positively regulate the mitogenic actions of IGF-I, Nck (adaptor protein) and also Grb2/Sos (Backer *et al.*1992, Xiao *et al.* 1994).

The physiological effects of IGF-I in many tissues, but specially in muscle, have been identified in both IGF-I null mice (Liu *et al.* 1993), which leads to skeletal muscle hypoplasia and death immediately after birth, and in IGF-I over-expressing transgenic mice, which have approximately 30% greater muscle mass than their non-transgenic littermates (Mathews *et al.* 1988). In addition to regulating myoblast proliferation through MAPK dependent pathways (LeRoith & Roberts 2003), IGF-I signaling also stimulates myoblast differentiation through the survival pathway mediated by PI3-K and eventually the induction of p21, a cyclin-dependent kinase (CDK) inhibitor. Like other CDK inhibitors, p21controls the CDKs by forming inhibitory complexes during the G1 and S phases of the cell cycle which stimulates cell cycle withdrawal (Lawlor & Rotwein 2000).

The activity and bioavailability of IGF-I and -II are extensively modulated by six highly homologous IGFBPs that have equal or slightly higher affinities for the IGFs than the type-I receptor (Yan *et al.* 2004). Systemically, the IGFBPs bind IGFs and prevent their clearance by increasing the circulating half-lives. They also regulate IGF-I and -II distribution and can either block or facilitate access to the receptor (Firth & Baxter 2002). All IGFBPs share highly conserved carboxyl and amino terminal domains and each contain a variable central linker domain (Clemmons 2001). The amino terminal domain is responsible for IGF binding while the carboxyl terminal cystein-rich domain is responsible for interacting with other proteins. The linker domain is often modified post-translationally in a manner specific to each IGFBP (Duan & Xu 2005). That either includes glycosylation (IGFBP-3, -4 & -5) and/ or phosphorylation (IGFBP-1, -3 & -5) (see figure 1). The former does not affect IGF binding affinity (Bach *et al.* 1992, Carr *et al.* 1994) but it increases IGFBP stability in part by decreasing susceptibility to proteolysis (Firth & Baxter 1999, Marinaro *et al.*2000). It is unclear how phosphorylation affects most IGFBPs. However, the IGF-I binding ability of IGFBP-1 increases six fold after phosphorylation (Jones *et al.*1991). By contrast, the affinity of phosphorylated IGFBP-3 for IGF-I is unchanged (Hoeck & Mukku 1994), although the growth inhibitory actions of IGFBP-3 are enhanced by phosphorylation (Hollowood *et al.*2002). This suggests that some of IGFBP-3's actions are independent of IGF binding and that phosphorylation of IGFBP-3 is involved.

In serum, about 75% of IGF-I is transported in a 150 kDa ternary complex with IGFBP-3 and the acid-labile subunit. The complex also forms with IGFBP-5, which is structurally very similar to IGFBP-3, although this occurs infrequently. The formations of

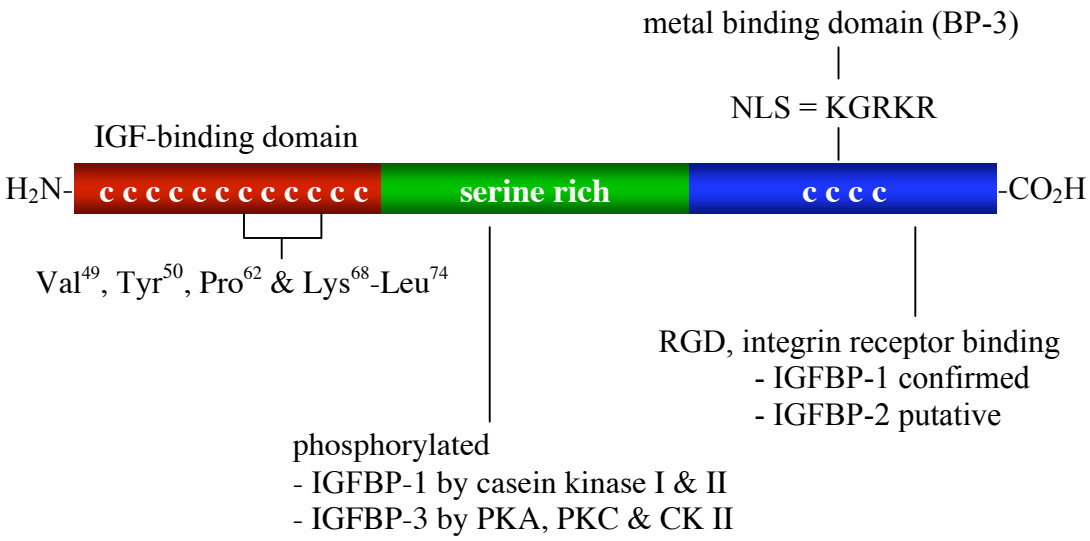


Figure 1. Anatomy of an IGFBP. There is 50% homology between different IGFBPs and 40-80% between different species. These differences are mainly come from two major post-translational modifications, glycosylation and phosphorylation, in the linker domain which give each binding protein possess specific function.

these complexes is primarily responsible for maintaining the circulating IGF-reservoir and in prolonging IGF half-life as only 5% of IGF-I circulates in a free and unbound state (Firth & Baxter 2002). The remaining 20% forms 40~50 kDa complexes with the remaining IGFBPs. These smaller complexes readily cross the vascular endothelium where they regulate IGF availability at the tissue and cellular level (Payet *et al.* 2004).

The modulation of IGF actions by "stimulatory" or "inhibitory" IGFBPs varies depending upon the cell type, the presence of other interacting proteins and experimental conditions. For example, IGFBP-4 and -6 have been consistently identified to inhibit IGF actions locally, although IGFBP-1, -2, -3 and -5 have been demonstrated to either stimulate or inhibit the effects of IGF-I (Duan & Xu 2005). When IGF-I and IGFBP-3 are co-incubated with human skin fibroblasts, IGFBP-3 inhibits the proliferative effects of IGF-I (Conover *et al.* 1996). Another study using primary human chondrocytes indicates that an IGF-I analogue des(1-3) IGF-I incapable of binding to IGFBPs is more potent in stimulating cell proliferation than normal IGF-I, which also suggests that endogeneously produced IGFBPs inhibit IGF-I actions locally (Matsumoto *et al.* 2000). By contrast, but using the same human skin fibroblast system, preincubating IGFBP-3 with IGF-I facilitates the binding interaction between IGF-I and its receptor and helps to stimulate the proliferation (De Mellow & Baxter 1988). The IGFBP-3 has the potential to decrease the IGF-I proliferation ability in human osteosarcoma cells which is IGF-I dependent by cloned QAYL-IGF-I, a mutated IGF-I with reduced affinity to IGFBP3 (Schmid *et al.* 2001). Another interesting study indicates the IGFBP-3 has differential physiological effects at high and low concentrations as it inhibits cell growth at low concentrations, but promotes growth at high concentrations (McCaig *et al.* 2002).

These regulatory actions are generally dependent upon ligand binding, however, recent research has shown that some IGFBPs have functions completely independent from their interactions with the IGFs. This is particularly true for IGFBP-3 which was first observed to possess anti-proliferation functions in chicken embryo fibroblasts, although this was demonstrated using an IGFBP-3 fragment that lacked the IGF-I binding domain (Lalou *et al.* 1996). Similar IGF-independent and inhibitory actions have since been described in many different cell types including prostate cancer cells (Cohen *et al.* 1994, Angelloz-Nicoud & Binoux 1995), fibroblasts (Cohen *et al.* 1993, Lalou *et al.* 1995), breast cancer cells (Oh *et al.* 1995), mesenchymal chondroprogenitor cells (Longobardi *et al.* 2003), renal carcinoma cells (Cheung *et al.* 2004) and skeletal muscle myoblasts (Foulstone *et al.* 2003, Pampusch *et al.* 2003, Xi *et al.* 2004). These functions have been identified for the most part by using different IGFBP-3 mutants and deletion constructs, such as the GGG (Buckway *et al.* 2001) and LLGQ (Yan *et al.* 2004) IGFBP-3 mutants and the 22-/25- kDa proteolyzed fragment (Lalou *et al.* 1996) that do not bind the IGFs, but somehow still inhibit their actions. Studies with Long-R3-IGF-I, a mutant IGF-I analogue incapable of binding to IGFBPs but fully capable of normal interactions with its receptors (Tomas *et al.* 1992), and with cells from IGF-IR knock out mouse embryos (Rajah *et al.* 1997) have also demonstrated IGF-independent growth inhibitory actions of IGFBP-3.

Although the GH/ IGF-I axis is the principle regulator of IGFBP-3 production in the liver and consequentially circulating levels of IGFBP-3, the gene expression of IGFBP-3 can be induced by several anti-proliferative and pro-apoptotic factors in many different cell types. This coincides with the well documented IGF independent growth

inhibitory and apoptotic functions of IGFBP-3. There are several apoptotic factors identified that exert their effect through regulating the mRNA and protein levels of IGFBP-3 including transforming growth factor (TGF)-β I in PEMC (Kamanga-Sollo *et al.* 2005), breast cancer cells (Fanayan *et al.* 2000), and prostate cancer cells (Cohen *et al.* 2000); myostatin in PEMC (Kamanga-Sollo *et al.*2005); retinoid acid in breast cancer cell (Gucev *et al.* 1996); tumor necrosis factor (TNF)-α in breast cancer cells (Rozen *et al.* 1998); tumor suppressor p53 in breast cancer cells (Schedlich & Graham 2002) and prostate cancer cells (Rajah *et al.* 1997); and 1,25 dihydroxyvitamin D3 in prostate cancer cells (Boyle *et al.* 2001). These studies suggest that IGFBP-3 is not only a typical binding protein, but that it serves as an anti-proliferation factor as well. Indeed, cells with high levels of IGF-I expression more readily transform into malignant cells (LeRoith & Roberts 2003) and IGFBP-3 is an anti-cancer IGF-I antagonist in many cancerous cells (Lee & Cohen 2002) including those from lung (Yu *et al.*1999), breast (Schedlich & Graham 2002), prostate (Hong *et al.* 2002) and colon (Ma *et al.* 1999). Although the specific inhibitory mechanisms have not been elucidated, recent studies have identified a novel apoptotic signaling pathway in F9 embryonal carcinoma cells. Nuclear IGFBP-3 binds the Nur77/ RXRalpha heterodimer and facilitates its translocation from the nucleus to the mitochondria. This results in the release of cytoplasmic cytochrome c, the activation of the pro-apoptotic factor caspase (Lee *et al.* 2005) and eventually apoptosis.

These anti-proliferative effects do not always result in apoptosis and have been associated with myoblast differentiation. In cultures of primary myosatellite cells from adult human skeletal muscle, the IGFBP-3 has higher secretion positively in respond to cell differentiation caused by both IGF-I and non-IGFBP binding Long-R3-IGF-I which

indicates IGFBP-3 may be involved in myoblast differentiation in both IGF-dependent/-independent manners (Foulstone *et al.* 2003). Besides, the mRNA and protein level of IGFBP-3 changes with differentiation processes in porcine embryonic myogenic cells (Johnson *et al.* 1999). Furthermore, IGFBP-3 antisense oligonucleotides similarly reduced IGFBP-3 production and myoblast differentiation (Foulstone *et al.* 2003). Combined, these studies suggest that IGFBP-3 is a multifunctional regulator of proliferation and differentiation in muscle cells.

Quite remarkably, IGFBP3 has been demonstrated to translocate from extracellular compartments into the nuclei of different cell types including opossum kidney cells (Li *et al.* 1997), human lung cancer cells (Jaques *et al.* 1997), keratinocytes (Wraight *et al.* 1998) and human breast cancer cells (Schedlich *et al.* 1998). This nuclear transportation is depend on the C- terminal nuclear localization sequence (NLS) and is not mediated by classical receptor endocytosis (Schedlich *et al.* 1998), but may involve caveolae-mediated endocytosis. (Lee *et al.* 2004). The precise mechanism of IGFBP-3 translocation is not known, however, it directly associates with Transferrin and its receptor (Lee *et al.* 2004). This suggests that one mechanism may include the "piggy back" of IGFBP-3 with Transferrin into the cell. Receptors for Transferrin are widely, but not ubiquitously, expressed in many tissues, which may explain IGFBP-3's unique ability to nuclear locate in many, but not all cell types (Lee *et al.* 2004). The ability to nuclear locate is shared by IGFBP-5 which is structurally homologous to IGFBP-3 and contains many conserved elements including a heparin binding motif (HBM) and a NLS within its carboxyl terminal domain. The anti-proliferative functions of IGFBP-3 are required for the TGF-β stimulated growth inhibition of some human breast cancer cells and these

effects are independent of IGF-I binding (Oh *et al.*1995, Kim *et al.* 2004). Besides breast cancer cells, IGFBP-3 also forms a complex with nuclear RXR-alpha inside the nucleus of LAPC-4 prostate cancer cells under the treatment of retinoid acid (Liu *et al.* 2000) and thereby stimulates apoptosis in an IGF-I-independent manner (Hong *et al.* 2002). By studying the apoptotic resistant mechanism of senescent fibroblasts, Hampel *et al.* (2005) reported that IGFBP-3 nuclear localization may be required for apoptosis in some cells. Although the functional role of nuclear IGFBP-3 is still unclear at present, these studies suggest that IGFBP-3 might exert its IGF-I independent anti-proliferative functions by controlling gene expression inside the nucleus. The nuclear localization of IGFBP-3 and -5 is a dynamic paradigm shift in fundamental peptide hormone signaling and suggests that some large hydrophilic peptides may be able to influence cellular processes without second messenger mediation. However, very few intracellular binding partners for either IGFBP-3 or -5 have been identified and clearly defined nuclear functions are also unknown. This illustrates the need for more functional studies into the unique and intracellular actions of these related proteins.

CHAPTER TWO

RNA POLYMERASE II (RPB3), A POTENTIAL NUCLEAR TARGET OF IGFBP-3

Introduction

IGF-I has been identified as a multifunctional cytokine in regulating both aspects of muscle development, proliferation and differentiation, which is dependent upon cell cycle progression and by contrast, growth arrest (Mathews *et al.* 1988, Liu *et al.* 1993). Recent studies suggest that IGFBP-3 may inhibit IGF-I stimulated myoblast proliferation by both IGF-I-dependent and -independent means (Pampusch *et al.* 2003), which in turn may influence differentiation. These include direct anti-proliferation effects in L6 myogenic cells and a mediating role in the TGF-β and myostatin-induced growth inhibition of porcine embryonic myogenic cells (Kamanga-Sollo *et al.* 2003). These effects complement the well known anti-proliferative effects of IGFBP-3 in several cancer cell lines which are independent of direct IGF binding, but dependent upon IGFBP-3 nuclear translocalization (Firth & Baxter 2002). The functional role, however, of nuclear IGFBP-3 in modulating skeletal muscle growth remains unclear. We therefore propose that IGFBP-3 could interact with nuclear targets to inhibit myoblast proliferation.

Materials and Methods

Stable transformation of Chinese hamster ovary (CHO) cells

The cDNA for a previously described non-IGF-binding human IGFBP-3 with glycine substitutions for residues critical to IGF-binding (I56, L80, and L81) was kindly

provided by Ron Rosenfeld. The mutant cDNA was subcloned from pCMV6-(GGG)BP-3 into the pcDNA3.1 expression vector creating pcDNA3.1-(GGG)BP-3. CHO cells were transfected with 5 μg pcDNA3.1 or pcDNA3.1-(GGG)BP-3 and LipofectAmine2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Stably transfected cells were derived in 600 μg/ml G418, and six monoclonal CHO/empty vector (EV) and CHO/(GGG)- IGFBP-3 lines were eventually expanded. The presence of mutant protein in the serum-free conditioned medium from three CHO/(GGG)IGFBP-3 clonal cell lines was verified by Western blotting with αhIGFBP-3 (Diagnostic Systems Laboratory, Webster, TX). In addition, serum free conditioned medium was collected from confluent CHO/(GGG)IGFBP-3 clone 1 cells after 48 h and the concentration of mutant protein was measured using a hIGFBP-3 enzyme-linked immunosorbant assay (Diagnostic Systems Laboratory).

Proliferation and apoptosis assays

Equal numbers of CHO/EV and CHO/(GGG)IGFBP-3 cells were grown to confluency in F-12K medium, which was then replaced with myoblast growth medium (DMEM/10% fetal bovine serum) for 24 h. This conditioned media was then used to replace the nonconditioned growth medium on myoblasts that were previously plated in 96-well plates and grown to approximately 50% confluency. Proliferation (Cell- Titer 96; Promega, Madison, WI) and apoptosis (caspase-3 and -7, ApoONE; Promega) assays were performed on these cells after 24 h according to the manufacturers' protocols. Proliferation assays were also performed on L6 myoblasts (50% confluent) cultured in serum-free DMEM supplemented with 1 nm Long-R3-(LR3) IGF-I (Diagnostic Systems

Laboratory) and 1.0 or 0.5 nm recombinant IGFBP-3 or the MDGEA IGFBP-3 that has a mutated NLS; ^{228}KGRKR to ^{228}MDGEA (Schedlich *et al.* 2000). Experiments were repeated twice (Fig. 1, n = 12/experiment; see Fig. 8, n = 7/experiment) and differences between groups were determined by a Student's t test or by an ANOVA coupled to Fisher's least significant difference test for multiple mean comparisons. Statistical analyses were performed on individual assays and on pooled data by expressing the arbitrary values as a percentage of controls (Fig. 1, n = 24; see Fig. 8, n = 14) and differences were detected using both approaches.

Fluorescent microscopy

Rat L6 myoblasts were plated in DMEM supplemented with 10% fetal bovine serum and grown to 50% confluency on coverslips. Cells were then cultured in the absence or presence of 500 ng/ml recombinant human IGFBP-3 (Protigen, Inc., Mountainview, CA) for 60 min. After washing three times in PBS, cells were then fixed in 1% paraformaldehyde on ice for 15 min, washed three additional times, and permeabilized on ice with 0.2% Triton X-100 for 15 min. Nonspecific binding sites were blocked by incubating cells in 3% normal goat serum diluted in PBS for 1 h at room temperature. Cells were subsequently probed with αhIGFBP-3 (1:200; Diagnostic Systems Laboratory) for 1 h at room temperature, washed three times and then similarly incubated with a fluorescein isothiocyanate (FITC)-conjugated rabbit antigoat secondary antibody (RAG^{FITC}, 1:300; Vector Laboratories, Burlingame, CA) in the dark. Cell nuclei were labeled by staining DNA with 5 μg/ml 4',6-diamidino-2- phenylindole (DAPI) for 2 min, also in the dark. Coverslips were then mounted with Fluoromount (Diagnostic

BioSystems, Pleasanton, CA) antifading reagent and sealed. Positive IGFBP-3 immunoreactivity and DNA staining of cells treated with hIGFBP-3 and of controls- untreated cells stained with both primary and secondary antisera or treated cells stained with secondary alone- was detected using fluorescent microscopy. In separate experiments, immunolocalization of exogenous IGFBP-3 was also determined as described using cells that were treated for 5, 20, and 60 min. In these experiments, IGFBP-3 immunoreactivity was visualized using a two-photon Leica (Bannockburn, IL) TCS SP confocal microscope.

To determine whether the MBD region of IGFBP-3, which also contains the NLS and caveolin binding box, possibly facilitates plasma as well as nuclear membrane translocation in these cells, myoblasts were incubated with 500 ng/ml GFP-chimeric peptides (Protigen, Inc.) that corresponded to either the carboxy-terminal MBD/NLS region (^{242}KKGFYKKKQCRPSKGRKRGFCW^{263}) or to residues 176–194 of IGFBP-3 (KKGHAKDSQRYKVDESQS, control peptide). Cells were terminated after 5, 20, and 60 min, fixed and DNA was stained as described. Cellular localization of the chimeras was then visualized by confocal microscopy. These were among many recently characterized IGFBP-3 peptide-GFP chimeras, GFP32 and GFP31, respectively (Singh *et al.* 2004).

Cellular fractionization and Western blotting

Replicate cultures of L6 myoblasts were grown to approximately 70% confluency and treated with or without 500 ng/ml IGFBP-3 for 60 min. Myoblasts were also grown to 50% confluency and stimulated to differentiate by serum withdrawal; replacing growth

medium with DMEM/ 2% horse serum. Myoblasts and fully differentiated myotubes were washed five times in PBS at room temperature and cytosolic and nuclear protein was isolated with the CelLytic NuCLEAR fractionation kit (Sigma, St. Louis, MO) according to the manufacturer's protocol. Protein concentration was determined by the Lowry assay (Bio-Rad, Hercules, CA) and 30 μg from each replicated fraction were separated by denaturing PAGE under reducing conditions (10% β-mercaptoethanol). Protein was transferred onto a 0.2 μm polyvinylidene difluoride membrane (Bio-Rad), which was subsequently blocked in 5% nonfat milk (Bio-Rad) prepared in 20 mm Tris.HCl (pH 7.5), 137 mm NaCl, and 0.1% Tween 20 (TBST). The membrane was then probed with αhIGFBP-3 (1:2000; Diagnostic Systems Laboratory) for 60 min in 5% milk, subjected to 3- to 10-min washes in TBST and reprobed with a RAG secondary for 30 min, all at room temperature. After repeating the washes, positive immunogenic reactions were visualized with enhanced chemiluminescence reagents in combination with Hyperfilm-ECL (both from Amersham Life Science, Arlington Heights, IL).

Yeast two-hybrid assays

Total RNA was isolated from L6 myoblasts with Trizol (Invitrogen) and mRNA was further isolated using oligo(dT) cellulose following the MicroPoly(A) Purist (Ambion, Austin, TX) protocol. Double-stranded cDNA was generated using Moloney murine leukemia virus reverse transcriptase and oligo(dT) primers with the BD SMART kit (BD Biosciences, Palo Alto, CA) and a cDNA library was constructed from PCR-amplified cDNA using the BD Matchmaker library construction and screening kit (BD Biosciences). Two separate bait vectors were constructed from the wild-type hIGFBP-3

cDNA, BP3/231 (231 amino acids, L61-K291) and BP3/111 (K181-K291), which were ultimately used in the screening assay. Flanking *Eco*RI and *Bam*HI sites were introduced by PCR (25 cycles of 94 C for 30 sec, 60 C for 30 sec, and 68 C for 1 min) using primers listed in Table 1. The resulting amplicons were then subcloned into pGBKT7 in-frame with the GAL4 DNA binding domain producing pGBKT7-BP3/231 and -BP3/111. Before screening, yeast (AH109) were transformed with both plasmids separately to ensure that neither induced reporter gene transactivation alone. Yeast were then cotransformed with the GAL4 activation domain pGADT7-Rec vector, double stranded cDNA and pGBKT7-BP3/231 or BP3/111 vectors and allowed to grow at 30C on high-stringency synthetic dropout (SD) plates lacking tryptophan, leucine, adenine, and histidine (Trp^-/ Leu^-/ Ade^-/ His^-). Colonies were collected and cultured in 3 ml of liquid SD/ -Trp/ -Leu/ -Ade/ -His for 24 h. Cells were harvested, resuspended in 100 μl of SD/ -Trp/ -Leu/ -Ade/ -His medium supplemented with 100 U of Lyticase (Sigma) and incubated at 37 C for 1 h under vigorous shaking. Twenty microliters of 20% sodium dodecyl sulfate were added to each sample and tubes vortexed vigorously. Samples were put through a freeze/thaw cycle at -20 C and vortexed to ensure complete cell lysis. Both pGADT7 and pGBKT7 recombinant plasmids were then isolated using the QIAprep miniprep kit (QIAGEN, Valencia, CA) and transformed into DH5α Escherichia coli strain. pGADT7 library plasmids containing cDNA for IGFBP-3-interacting proteins were amplified and separated from pGBKT7 vectors by growing transformed DH5α in the presence of ampicillin alone. Different IGFBP-3 and Rpb3 deletion mutants were similarly constructed using PCR as described above (see Table 1) and used to confirm the

Table1. Subcloning primers for IGFBP-3 and Rpb3 deletion mutants

Name	Forward primer (5'–3')	Reverse primer (5'–3')
Rpb3/120	CATGCCATGGTGATGCCGTACGCCAACCAG	GCGGATCCGAGATCTCGGGAGGTAACATG
Rpb3/158	GCGAATTCCGAGATCTCATCTCCAACAGC	GCGGATCCAACCTAGTCTCCGCAGCAGGC
hBP3/231	CGGAATTCCTGGTGCGCGAGCCGGGCTGC	CGGGATCCCTTGCTCTGCATGCTGTAGC
hBP3/111	CGGAATTCAAAGACAGCCAGCGCTACAAAG	CGGGATCCCTTGCTCTGCATGCTGTAGC
hBP3/39	GCGAATTCCCCAGGGGTGTACACATTCC	GCGGATCCCTGCCCATACTTATCCACACAC

Rpb3/120, Amino acids M1-L120; Rpb3/158, R118-N275; hBP3/231, L61-K291; hBP3/111, K181-K291; and hBP3/39, P232-P270.

library screening results as well as to identify the interacting domains. These include BP3/39 (P232-P270) subcloned into pGBKT7 and Rpb3/120 (M1-L120) and Rpb3/157 (R118- N275) into pGADT7. Such forced interactions were performed by first growing transformed yeast on Trp^-/ Leu^- plates and by restreaking colonies on Trp^-/ Leu^-/ Ade^-/ His^- plates.

Coimmunoprecipitation assays

Rpb3 (purchased under the commercial name of POLR2C; USBiological, Swampscott, MA) was incubated for 3 h on a rotary shaker at room temperature with either the recombinant IGFBP-3 or the MDGEA-IGFBP-3 mutant in 500μl of the binding buffer (150 mm NaCl, 1 mm MgCl2, 1 mm CaCl2 and 0.5% CHAPS in 1X PBS) and at a concentration of 1μg/ml each. Antibodies against either IGFBP-3 (rabbit anti-IGFBP-3 IgG; Santa Cruz Biotechnology, Santa Cruz, CA) or Rbp3 (chicken anti- POLR2C IgY; ProSci Inc., Poway, CA) were added at titers of 1:50 and 1:200, respectively, and the reactions incubated for an additional 1 h. When chicken anti-POLR2C antibodies were used, rabbit antichicken IgY antibodies (Immunology Consultants Laboratory, Newberg, OR) were added (1:200) for an additional 1 h incubation (IgY type antibodies are not recognized by proteins A or G). Protein/antibody complexes were precipitated using 50μl of Protein G Plus/Protein A-Agarose beads (Calbiochem, San Diego, CA) prewashed with the binding buffer. Precipitated complexes were washed three times with fresh binding buffer, solubilized in Laemmli Loading buffer and boiled for 10 min. Fractions were loaded on a SDS-PAGE gel and immunoblotted for the presence of IGFBP-3, the MDGEA-IGFBP-3 mutant, or of Rpb3. Binding interactions between exogenous IGFBP-

3 and endogenous Rpb3 were also investigated using nuclear lysates. Proliferating L6 myoblasts (~80% confluent) were incubated with or without 1μg/ml IGFBP-3 for 60 min. Cells were washed thoroughly, and nuclear protein was extracted using the Nuclear Complex Co-IP Kit according to the manufacturer's protocol (Active Motif, Carlsbad, CA). To preserve potentially unstable protein/protein interactions, enzymatic shearing was performed at 4 C for 90 min instead of the standard 10 min at 37 C. Nuclear protein was immunoprecipitated as described above using the manufacturer's buffer and protocol with the exception of the following modifications: each reaction included 500 μg of nuclear protein mixed with or without 5μg of polyclonal αIGFBP-3 (Santa Cruz Biotechnology) and both the incubation and the washing buffers were supplemented with 150 mm NaCl and 0.5% CHAPS, but did not contain dithiothreitol. Immunoprecipitated proteins were then detected by immunoblotting as described with either αIGFBP-3 or αRpb3.

Results

IGF-independent inhibition of myoblast proliferation

The presence of mutant protein in the conditioned medium of three CHO/(GGG)IGFBP-3 cell lines was confirmed by Western blotting (Fig. 2A) and was additionally measured in the serum-free conditioned medium of the first clonal line by an ELISA (338.5 ng/ml). Conditioned myoblast growth media (DMEM/10% fetal bovine serum) from control CHO cells stably transfected with an empty vector (CHO/EV) or from CHO/(GGG)IGFBP-3 clone 1 were then used to determine the IGF-independent

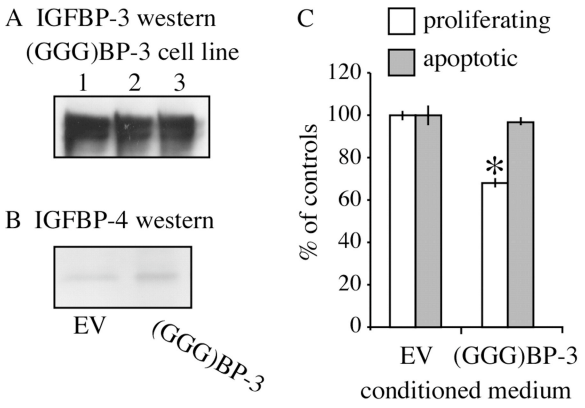


Figure 2. IGF-independent inhibition of myoblast proliferation by IGFBP-3. (A) Western immunoblotting of conditioned media from three stably transfected CHO cell lines overexpressing a non- IGF-binding IGFBP-3 termed (GGG)BP-3. (B) Detection of IGFBP-4 in conditioned media of CHO cells transfected with an empty vector (EV) or with (GGG)IGFBP-3. Media from confluent cells were concentrated 10-fold by spin filtration and the end volumes were normalized. An equal volume from each medium was then analyzing by western blotting with αIGFBP-4. (C) Conditioned medium from CHO cells transfected with an empty vector or from CHO/(GGG)BP-3 cells was added to proliferating myoblasts for 24 hr. Cell number and apoptosis assays were performed as described in Materials and Methods. The arbitrary values from both proliferation and apoptosis assays are expressed as a % of controls, CHO cell conditioned medium values (n=24). ($p<0.05$).

effects of IGFBP-3 on myoblast proliferation. The number of proliferating myoblasts cultured for 24 h with medium containing (GGG)IGFBP-3 was approximately 35% less than cells cultured with CHO/EV conditioned medium (Fig. 2C). Thus, IGFBP-3 directly inhibited myoblast proliferation in vitro without necessarily sequestering locally produced IGF-I or that contained within the serum. This effect was not due to plating efficiency or to apoptosis because caspase-3/7 activity was identical in both groups, suggesting that the (GGG)IGFBP-3-induced reduction in myoblast cell number was due to either a reduced proliferation rate or to cell cycle growth arrest, but not to cell death. CHO cells express predominantly IGFBP-4, which were determined to be equal in conditioned medium from both CHO/EV and CHO/(GGG)IGFBP-3 cells (Fig. 2B). Levels of IGFBP-4 were in fact identical in media from all CHO/ (GGG)IGFBP-3 clonal cell lines despite sometimes very different levels of mutant IGFBP-3 (data not shown). Therefore, the over expression of (GGG)IGFBP-3 does not result in a compensatory increase in IGFBP-4 secretion. These results additionally suggest that the growth inhibitory effects of the CHO/(GGG)IGFBP-3 medium was due to the mutant IGFBP-3 and not to changes in IGFBP-4 production.

Nuclear localization of recombinant IGFBP-3 in rat L6 myoblasts

IGFBP-3 immunoreactivity was absent in control cells incubated without recombinant protein and stained with both primary and secondary antisera (Fig. 3, middle panel) and was similarly lacking in control cells incubated with 500 ng/ml IGFBP-3, but stained with secondary antibody alone (Fig. 3, left panel). Thus, these primary and secondary antisera do not cross-react with nonspecific proteins under these conditions. In

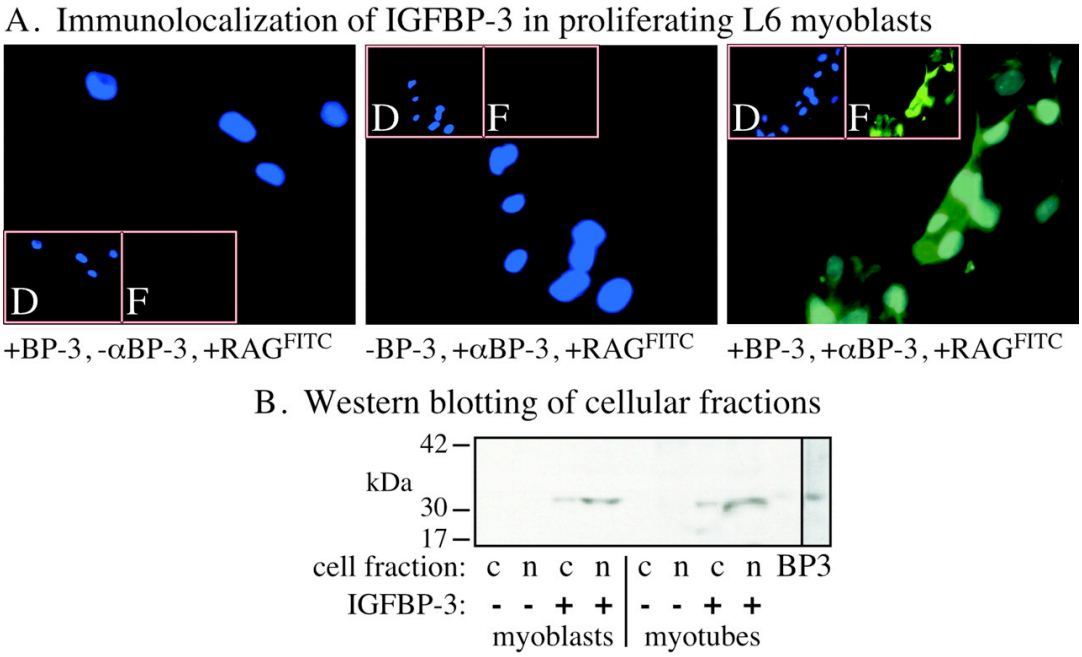


Figure 3. IGFBP-3 nuclear localization in L6 myoblasts. (A) Cells were incubated -/+ 500 ng/ml IGFBP-3 for 1 h, fixed and stained -/+ anti-human IGFBP-3 (αBP-3) and -/+ FITC-conjugated rabbit anti-goat secondary (RAGFITC). Blue DAPI staining of nuclei (D) and green RAGFITC staining of IGFBP-3 (F) images are inset of overlay images. Co-localization/staining of DNA and IGFBP-3 is indicated by turquoise. Left and middle panels indicate no non-specific staining of secondary and primary antisera, respectively. (B) Western blotting of cellular fractions from L6 myoblasts & myotubes incubated with IGFBP-3. Myoblasts and fully differentiated myotubes were incubated -/+ 500 ng/ml IGFBP-3 for 60 min. Cytosolic (c) and nuclear (n) fractions were then blotted with hIGFBP-3. (BP-3 = short & long exposures of 20 ng IGFBP-3 peptide)

cells treated with recombinant protein and stained with both antisera, IGFBP-3 immunoreactivity was located within myoblast nuclei as indicated by overlapping staining of DNA (blue) and IGFBP-3 (green) (Fig. 3, right panel). These results suggest that exogenous IGFBP-3 translocates across the plasma and nuclear membranes of proliferating myoblasts in vitro and ultimately nuclear locates within the 60-min culture period. Cells were fixed in paraformaldehyde and washed extensively (six times total) before permeabilization. Therefore, nuclear localization of IGFBP-3 is not due to contamination of nuclear and other intracellular compartments during the immunolabeling procedure. Plasma membrane and/or cytosolic staining was also detected in these cells (Fig.3, right panel) but could not be distinguished from one another using normal fluorescent microscopy. Subsequent studies were, therefore, performed using a confocal microscope. Western blotting of equal amounts of cytosolic and nuclear protein from cells incubated with IGFBP-3 for 60 min independently verified the presence of IGFBP-3 in the nucleus (Fig. 3B). The protein was intact in both cellular fractions and was not proteolyzed. Thus, the in situ immunofluorescence of IGFBP-3 in the former assays is not due to immunoreactive byproducts of IGFBP-3 degradation. Absolute levels of IGFBP-3 were greater in nuclear protein fractions than in cytosolic, which is consistent with the transitional movement of IGFBP-3 from the cytosol to the nucleus. Intact IGFBP-3 was additionally detected in nuclear protein of fully differentiated myotubes as well as in proliferating myoblasts, which suggests that a nuclear role for IGFBP-3 is preserved even after differentiation.

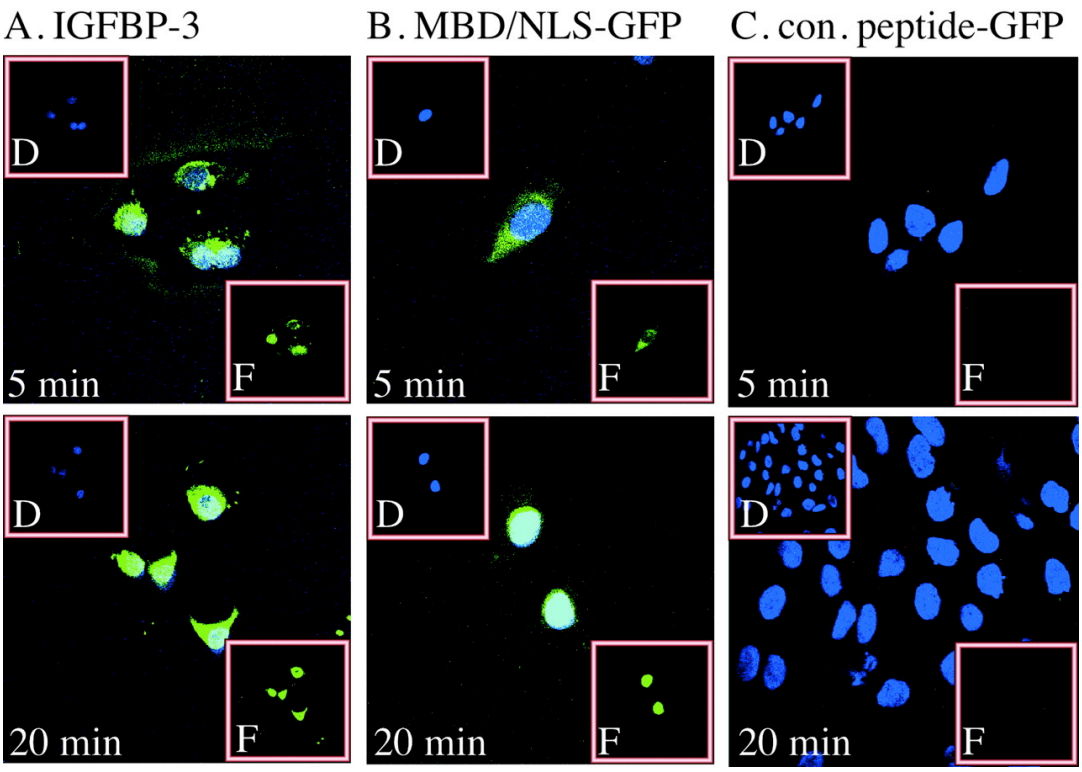


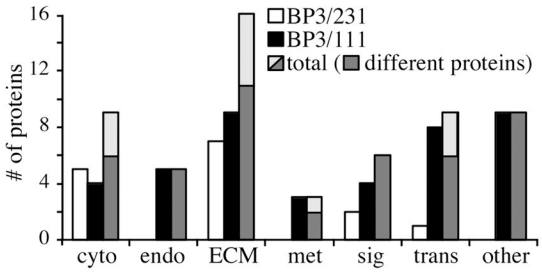
Figure 4. IGFBP-3 and MBD peptide time course for nuclear localization in L6 myoblasts. (A column) Confocal microscopy of myoblasts incubated with 500 ng/ml IGFBP-3 for 5 and 20 min. Control cells incubated in the absence of IGFBP-3 were negative (data not shown). (B column) Myoblasts incubated with 500 ng/ml of a GFP-chimeric peptide that corresponds to the MBD/NLS domain of IGFBP-3 (MBD/NLS-GFP, residues 242-263) or to (C column) a control peptide (residues 176-194 of IGFBP-3). Images of DAPI-stained nuclei (D) and RAGFITC-stained IGFBP-3/GFP fluorescent (F) are inset of overlay images.

IGFBP-3 nuclear localization time-course

Immunolocalization of IGFBP-3 was determined in cells treated for 5 and 20 min (Fig. 4A). A minimal amount of exogenous IGFBP-3 was located within the nuclei after only 5 min as the majority of immunoreactivity was cytosolic and perinuclear. By contrast, both cytosolic and nuclear compartments were entirely engulfed with IGFBP-3 after just 20 min. This rapid pattern of translocation was mimicked by the GFP chimera containing the MBD/NLS peptide (Fig. 4B), but not by the control chimera (Fig. 4C). These results suggest that the MBD/NLS region of IGFBP-3 may facilitate the crossing of both plasma and nuclear membranes. They further suggest that a NLS/MBD peptide could similarly direct other proteins or large hydrophilic molecules across these membranes and into the cytosol and/or nuclei of myoblasts and possibly other cell types. The identification of nuclear IGFBP-3 using fluorescent microscopy, confocal microscopy, and Western blotting all suggest, moreover independently verify, that IGFBP-3 rapidly translocates to myoblast nuclei.

Identification of IGFBP-3-interacting proteins

A total of 57 clones (12 duplicates) were identified as putative IGFBP-3-interacting proteins after screening a custom L6 myoblast cDNA expression library using a high stringency yeast two-hybrid assay (Fig. 5) and two different IGFBP-3 deletion mutants as bait: BP3/231 and BP3/111 (Fig. 6). Fifteen clones were isolated with BP3/231 and 42 with BP3/111, possibly indicating steric hindrance by the N-terminal region. Thirteen proteins have been previously shown to directly interact with IGFBP-3, and three of these were also identified in our screens, including fibronectin, type Iα-



Previously identified IGFBP-3-interacting proteins (excluding IGF-1 & -II).

protein	class#	method*	source	ref.
heparin	ECM	affi	recombinant	26
fibrinogen/fibrin	ECM	affi	fibrin clots	27
fibronectin	**ECM**	**Y2H**	**placenta**	28
type I α-collagen	**ECM**	**Y2H/affi**	**serum/liver**	29
type V TGFβ receptor	endo	affi	lung empthelium	30, 31
transferrin	endo	Y2H/affi	liver/serum	21
latent TGFβ BP	endo	Y2H	embryo	32
humanin	endo	Y2H	prostate	33
IGFBP-3	**endo**	**affi**	**breast (T47D cells)**	34
AMF/PGI	endo	affi	breast (T47D, MCF-7)	35
ADAM 12	protease	Y2H	placenta	36
RAR	signaling	Y2H	breast (Hs578T)	37
RXRα	signaling	Y2H	prostate	17

Figure 5. Identification of IGFBP-3-interacting proteins in L6 myoblasts. A custom cDNA expression library was screened using a high stringency yeast 2-hybrid assay (BD Bioscience) and two different IGFBP-3 deletion mutants as "bait": BP3/231 and BP3/111 (see Figure 5). A total of 57 clones were identified, 15 with BP3/231 and 42 with BP3/111. These include 12 duplicates as well as 3 previously identified IGFBP-3-interacting proteins (table). The functional distribution of each protein isolated with the indicated bait construct is shown in the histogram (cyto, cytosolic; endo, endocrine; ECM, extracellular matrix; met, metabolic; sig, signaling; trans, transcription). Previously identified IGFBP-3-interacting proteins are shown in the table, including those also identified in our assays (bold). *method of detection = yeast 2-hybrid screening (Y2H), ligand affinity/chromatography (affi); #functional class = extracellular matrix (ECM), endocrine or related (endo)

collagen and IGFBP-3 itself, which independently validates our assay (Fig. 5). Several diverse functional classes were represented among the clones, although extracellular matrix proteins were best represented with 16 total clones (11 different proteins and five duplicates). Most interesting, however, was the identification of a nuclear pore protein as well as six different proteins involved in transcription including three clones of the rat homolog for RNA polymerase II (RNAPII) binding subunit 3 (Rpb3, a.k.a. subunit C, GenBank accession no. NP_001012491) and two clones of a structurally similar subunit of RNA polymerase I (NP_001008331).

To confirm the binding interactions of BP3/ 231 and BP3/ 111 with Rpb3, yeast two-hybrid assays were performed using yeast cotransfected with either the full-length Rpb3 or the negative control activation domain vector, simian virus 40 (SV40), in combination with BP3/231, BP3/111 or with a negative control DNA-binding domain vector, Lamin C. With each transfection, the presence of both DNA-binding and activation domain vectors was verified by conditional growth on Leu^-/ Trp^- plates (Fig. 6). Cells overexpressing either BP3/231 or BP3/111 and Rpb3 grew after replating on Leu^- /Trp^- /His^- /Ade^- plates, which confirmed Rpb3 binding to these regions of IGFBP-3. However, neither of the negative controls grew under these conditions. Similar results were also obtained using RNA polymerase 1–1 (Pol-I; Fig. 6), which is structurally similar to Rpb3. This suggests that the Rpb3/IGFBP-3 binding interaction is specific within the limitations of this assay. To help identify the interacting domains, assays were also performed on yeast cotransfected with a smaller construct that codes for the 39 amino acid epitope containing the MBD/NLS (BP3/39) in combination with constructs for either the first 120 amino acids of Rpb3 (Rpb3/120) or the last 157 (Rpb3/157).

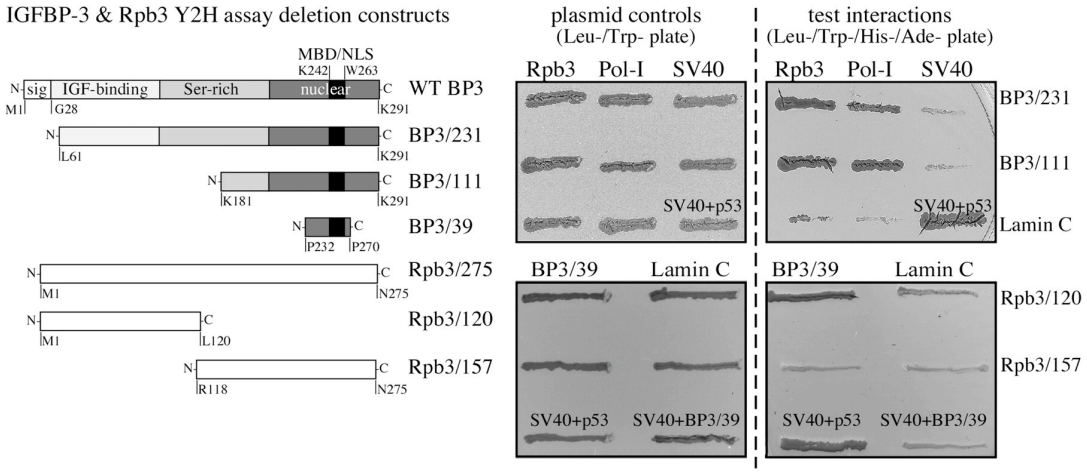


Figure 6. Binding interactions between IGFBP-3 & a RNA polymerase II subunit, Rpb3. RNA Polymerase II binding subunit 3 (Rpb3) and the homologous protein from the RNA Polymerase I complex (Pol-I) were identified in the Y2H screen, both with BP3/111. These interactions were confirmed by co-expressing the indicated proteins in Y2H assays. The putative Rpb3-binding domain within IGFBP- 3 was also identified using these assays. (left) Cartoon of IGFBP-3 and Rpb3 deletion mutants. Functional domains of IGFBP-3 are shaded and annotated and each IGFBP-3 and Rpb3 mutant is named based on the total number of residues within each mutant (WT, wild-type). (right) Yeast were co-transfected with plasmids containing the cDNA for the indicated mutants (IGFBP-3 in pGBKT7 DNA-binding vector; polymerases in pGADT7-Rec[2] activation domain vector) and spread upon leucine/tryptophan deficient plates (Leu^-/Trp^-). Only cells containing both plasmids will grow under these conditions. Individual colonies were also cultured under restrictive conditions ($Leu^-/Trp^-/His^-/Ade^-$ = histidine & adenine deficient as well) where growth requires the interaction between both proteins and the consequential expression of reporter genes. (SV40+p53 = positive control; Lamin C and SV40 = negative controls)

Conditional growth only occurred in cells overexpressing BP3/39 and Rpb3/120, indicating that binding occurs between the N-terminal half of Rpb3 and the region of IGFBP-3 that contains the MBD/ NLS epitope.

NLS requirement for IGFBP-3/Rpb3 binding and IGFBP-3-stimulated growth inhibition

The direct association of IGFBP-3 with Rpb3 was also confirmed using co-immunoprecipitation assays and antisera specific for each recombinant protein. When co-incubated, western blotting revealed the presence of both proteins in the αIGFBP-3 and αRpb3 immunoprecipitates (Fig. 7) and there was no indication of antibody cross-reactivity (left & middle panels, last lanes). By contrast, only a minimal amount of Rpb3 was detected within the αIGFBP-3 immunoprecipitates when Rpb3 was incubated with the MDGEA mutant instead of wild-type IGFBP-3 (right panel, lane 2 vs. 3). These data suggest that an intact NLS is required for significant IGFBP-3/Rpb3 binding. This interaction was also confirmed by coimmunoprecipitating nuclear lysates from cells treated exogenously with wild-type IGFBP-3 for 60 min (Fig. 8). Both IGFBP-3 and endogenous Rpb3 were detected in the precipitates indicating coassociation of these two proteins within nuclear lysates. The presence of Rpb3 was not due to non-specific interactions with the antiserum or with the protein A/G agarose as precipitates from cells treated without IGFBP-3 or lysates incubated with agarose alone did not contain Rpb3 (Fig. 8, last two lanes in both panels).

Proliferation assays were also conducted on myoblasts cultured for 48 h with LR3 IGF-I and either IGFBP-3 or the MDGEA mutant. As expected, LR3 IGF-I alone increased cell growth by almost 50% compared to controls (Fig. 9). Both doses of

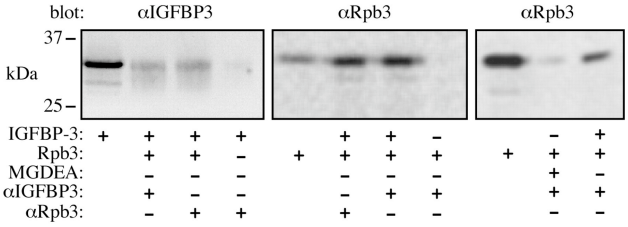


Figure 7. IGFBP-3/Rpb3 co-immunoprecipitation requires an intact NLS domain. Recombinant IGFBP-3 or the NLS-mutant that does not nuclear locate (MDGEA, see Firth & Baxter (5)) were incubated with recombinant Rpb3 and immunoprecipitated as described in the Materials and Methods with either IGFBP-3 or Rpb3 antisera as indicated. Precipitated complexes were washed, solubilized in SDS-PAGE loading buffer and immunoblotted with the indicated antisera. Antibody specificity was controlled by immunoprecipitating IGFBP-3 with αRpb3 or vice versa (left & middle panels, last lanes).

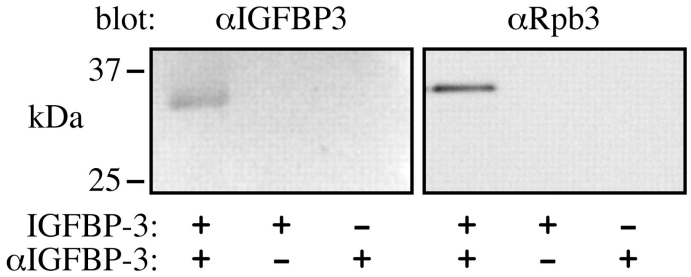


Figure 8. IGFBP-3/Rpb3 co-immunoprecipitation from L6 myoblast nuclear lysates. Proliferating cells were treated with or without recombinant IGFBP-3 for 60 min, washed thoroughly and nuclear lysates were isolated as described in the Materials and Methods. Equal amounts of nuclear protein from each treatment group were immunoprecipitated with αIGFBP-3 as indicated and samples were blotted as in Fig. 7. Non-specific binding of nuclear protein to the protein-A/G agarose or to IGFBP-3 was controlled by the inclusion of samples lacking either IGFBP-3 antibodies or peptide.

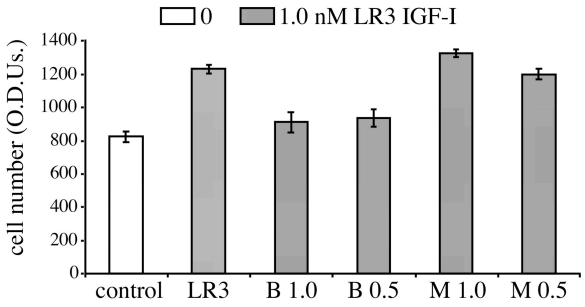


Figure 9. IGFBP-3 inhibition of myoblast proliferation requires an intact NLS domain. Proliferating L6 myoblasts were incubated for 48 h in serum-free DMEM with or without 1.0 nM LR3 IGF-I (does not recognize IGFBPs) in the presence of 1.0 and 0.5 nM wild-type IGFBP-3 (B) or the MDGEA mutant IGFBP-3 (M), which is incapable of nuclear localization (5). Cell number was determined using the CellTiter 96 colorimetric assay (Promega) according to the manufacturers' protocol (mean values + SEM are shown; n=14/treatment).

IGFBP-3, however, attenuated these effects, which confirm previous studies indicating a growth inhibitory role IGFBP-3 in these cells as well as the data presented in Fig. 2. This inhibitory effect was lost when cells were treated with the MDGEA mutant IGFBP-3. Thus, IGF-independent growth inhibition of myoblast proliferation requires IGFBP-3 nuclear localization and possibly Rpb3 binding.

Discussion

Incubating myoblasts with IGF-I has a dual effect on the cell cycle as the growth factor simultaneously stimulates proliferation and differentiation (Adams 2002). It also stimulates the synthesis and secretion of IGFBP-3 as cells begin to differentiate, a process that appears to be significantly influenced by IGFBP-3 (Foulstone *et al.* 2003, Johnson *et al.* 2003, Pampusch *et al.* 2003). We have shown that an IGFBP-3 analogue with no appreciable affinity for either IGF-I or -II can inhibit myoblast proliferation under growth conditions, which is the first step in the initiation of differentiation. Similar roles have been defined for IGFBP-3 in negatively regulating myoblast growth using primary human (Foulstone *et al.* 2003) and porcine (Johnson *et al.* 2003, Pampusch *et al.* 2003) myosatellite cells as well as rat L6 myoblasts (Xi *et al.* 2004) that incorporate both IGF-dependent and -independent mechanisms. Thus, IGF stimulated myoblast differentiation appears to be mediated, at least in part, by the local production of IGFBP-3, which serves a similar role for TGFβ1 and myostatin (Kamanga-Sollo *et al.* 2003).

Myoblast nuclear localization of IGFBP-3 was independently demonstrated in separate experiments using fluorescent and confocal microscopy and by western blotting of nuclear protein. The translocation of intact IGFBP-3 across both plasma and nuclear

membranes was rapid and occurred within 5 min, which is consistent with a functional nuclear role for IGFBP-3 in these cells and suggests that nuclear immunoreactivity was not due to non-specific endocytosis and subsequent proteolysis of IGFBP-3. The presence of IGFBP-3 within the nuclei of many different cell types in addition to rat myoblasts and myotubes (Jaques *et al.* 1997, Li *et al.* 1997, Schedlich *et al.* 1998, Wraight *et al.* 1998, Liu *et al.* 2000) suggests that IGFBP-3 helps in regulating fundamental cellular processes. However, IGFBP-3 stimulates apoptosis in many of these cells, but had no effect on the myoblasts described herein. This suggests that although the mechanisms required for IGFBP-3 nuclear entry may be relatively common, its function is likely cell type-specific and dependent upon the unique expression of intracellular and nuclear proteins. Some IGF-independent effects of IGFBP-3 are dependent upon its nuclear localization, which may include myoblast growth inhibition. Indeed, the MDGEA mutant IGFBP-3, which does not nuclear locate, is incapable of inhibiting LR3 IGF-stimulated proliferation (Fig. 9) and does not associate with Rpb3 in coimmunoprecipitation assays (Fig. 7). We have additionally demonstrated exogenous IGFBP-3 binding to endogenous Rpb3 in nuclear lysates (Fig. 8). These data suggest that nuclear localization and possibly Rpb3 binding, two related yet possibly exclusive events, are both necessary prerequisites for the IGF-independent inhibition of myoblast growth. The NLS domain is necessary for a number or binding interactions including the association of IGFBP-3 with importin-β (Firth & Baxter 2002, Schelich *et al.* 2000). However, the NLS is also a highly and negatively charged domain that when mutated, may alter the three dimensional structure of the C-terminal region, which would explain its dependence for so many binding interactions.

The first intracellular protein demonstrated to directly bind IGFBP-3 was RXRα (Liu *et al.* 2000), although Schedlich et al. 2004 recently demonstrated retinoic acid receptor (RAR) binding as well. Binding to RXRα enhanced transcriptional activity and apoptosis in the former study, whereas RAR binding specifically inhibited ligand activation and the formation of RXR:RAR heterodimers in the latter. Thus, a minimum role for IGFBP-3 in the nucleus appears to include the modulation of transactivation for some RXR- and RAR-responsive genes, which in turn would have different effects depending upon the cell type and the presence of different retinoid receptors. Gene expression requires recruitment of the RNAPII complex whose core enzyme is composed of 12 different subunits. Rpb3 is the third largest subunit and is located externally away from the core DNA-binding domain (Cramer 2004). Assembly of the RNAPII complex is dependent upon Rpb3, although its functional role, as is currently known, is to facilitate recruitment of RNAPII to specific transcription factors, which in turn initiates gene transcription (Tan *et al.* 2000, Corbi *et al.* 2002, De Angelis *et al.* 2003). Thus, nuclear IGFBP-3 may not need to bind DNA directly to influence transcription as it could assist in RNAPII recruitment to transcription factor complexes, including those involving RXR and RAR. Rpb3 is ubiquitously expressed in all tissues, however, its levels are considerably higher in cardiac and smooth muscle than in any other tissue (Fanciulli *et al.* 1996, 1998), which is suggestive of an alternative and muscle-specific role. Indeed, Rpb3 binds myogenin and activating transcription factor (ATF) 4 and directly facilitates their transactivational activity while simultaneously stimulating myoblast differentiation (Corbi *et al.* 2002, De Angelis *et al.* 2003). Corbi et al. 2005 recently demonstrated Rpb3 shuttling between cytoplasmic and nuclear compartments. Rpb3 association with HCR

(α-helix coiled-coil rod homologue) in the cytoplasm prevents nuclear entry and myoblast differentiation. Thus, a functional role for IGFBP-3 in regulating myoblast differentiation could include the "delivery" of Rpb3 to nuclear targets.

Lee et al. recently determined that cellular internalization of secreted IGFBP-3 is mediated by its binding to transferrin and to caveolin. Although internalization, nuclear entry and the apoptotic effects of IGFBP-3 were all completely blocked by inhibitors of caveolae formation, a minimal amount of a non-transferrin-binding mutant IGBFP-3 (K228E/R230G) still localized to the nucleus. In a similar study, Singh et al. characterized several peptides based on an IGFBP-3 epitope within its C-terminal domain and defined critical motifs within these GFP-chimeric peptides necessary for cellular internalization. Chimeras containing the transferrin- and partial caveolin-binding motifs all localized to the nucleus whereas those that lacked both motifs (GFP34) or the partial caveolin-binding motif alone (GFP36) remained extracellular. Co-immunoprecipitation, cross-linking and/or ligand blotting assays were used to demonstrate direct binding between IGFBP-3 or the MBD/NLS peptide with caveolin and either transferrin or its receptor (Lee *et al.* 2004, Singh *et al.* 2004). Together, these studies suggest that IGFBP-3 internalization is mediated by transferrin and caveolin, both of which are expressed in skeletal muscle cells including L6 myoblasts (Shimo-Oka *et al.* 1986, Scherer *et al.* 1997), and that caveolin-binding in particular is critical.

Previous studies with transformed skeletal muscle cell lines suggested that IGFBP-5 was the principle IGFBP secreted by muscle cells (Florini *et al.* 1996). By contrast, recent studies with primary skeletal muscle stem cells from human and porcine sources suggest that IGFBP-3 is specifically involved in the growth regulation of PEMC,

human skeletal muscle cells, and L6 myogenic cells. We hypothesize that IGFBP-3 partially mediates IGF-stimulated myoblast differentiation by inhibiting cell growth and that nuclear localization of IGFBP-3 is critical to this process (Fig. 9). Future studies will further explore the ability of IGFBP-3 to initiate differentiation and to regulate gene expression via its association with Rpb3. They will also determine if other proteins identified in our screening assay contribute to or enhance these effects. Nevertheless, these and other recent studies strongly suggest that skeletal muscle development is influenced by the IGF-independent actions of IGFBP-3 and that nuclear localization is required.

CHAPTER THREE

COMMERCIAL AND APPLIED ASPECTS OF IGFBP-3 THERAPEUTICS

Summary

Previous studies, including those discussed herein, indicate that IGFBP-3 serves as a multifunctional growth regulator in different cell types. Using non-IGF binding analogs of IGFBP-3, we confirmed that IGFBP-3 directly inhibits myoblast proliferation in an IGF-independent manner and that the NLS sequence is important for this function. We observed the rapid translocation of IGFBP-3 into L6 myoblast nuclei using various techniques. A GFP-NLS chimera also possesses the same rapid nuclear translocation ability and suggests that the NLS peptide could help deliver hydrophobic therapeutics to muscle cell nuclei. We additionally screened a custom cDNA library derived from L6 myoblasts using a Yeast 2-Hybrid assay and IGFBP-3 as bait. Herein, we identified several interacting proteins, most notably Rpb3, which was confirmed with coimmunoprecipitation assays. We also determined that the NLS sequence is important for IGF-independent myoblast growth inhibition. Several additional IGFBP-3 analogues have been generated by site-directed mutagenesis and will help in future studies seeking to better define the IGFBP-3/ Rpb3 binding interaction. We therefore present a model (Fig. 10) where the local production of IGFBP-3 inhibits myoblast proliferation in an IGF-independent manner. This requires an intact NLS and its rapid translocation of IGFBP-3 into the nucleus where it interacts with RNAPII via Rpb3. We hypothesize that

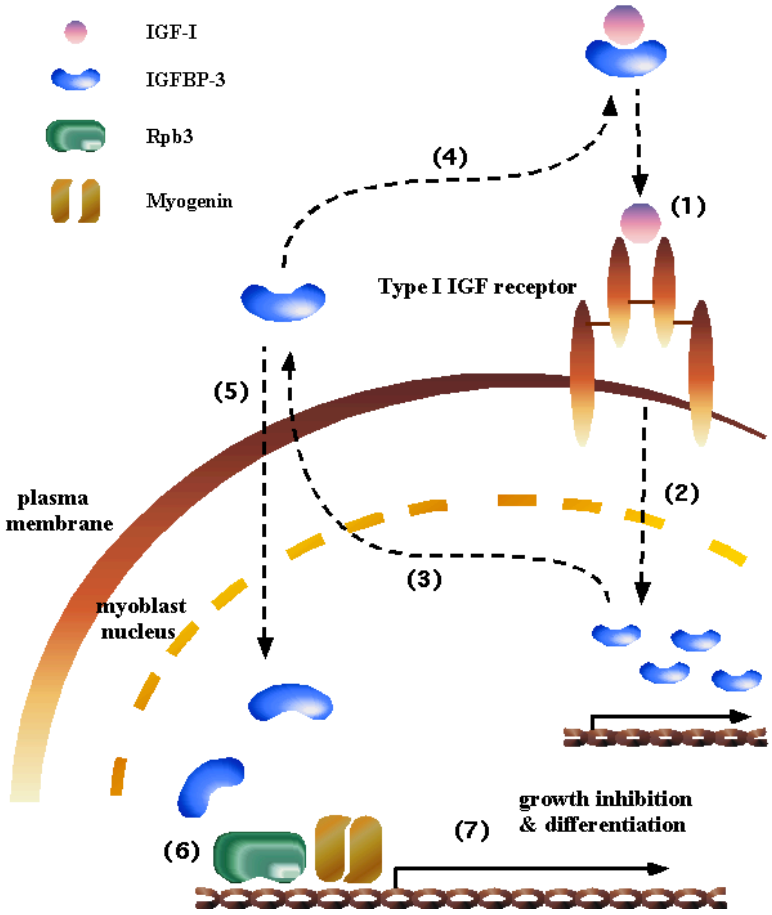


Figure 10. Model of IGFBP-3 regulated myoblast growth inhibition and subsequent differentiation. (1) IGF-I binds and activates its receptor, which (2) signals to the nucleus and stimulates IGFBP-3 gene expression and subsequently protein secretion (3). IGFBP-3 then binds IGF-I (4) thereby removing the mitogenic signal. It also translocates to the nucleus (5) where it binds Rpb3 (6) and influences the expression of myogenin-regulated genes (7). This in turn suppresses proliferation and initiates differentiation.

this interaction enhances the transactivational activity of two myoblast differentiation factors- myogenin and ATF-4- and in turn inhibits proliferations.

Medical and Veterinary implications

Several valuable studies of IGFBP-3's potential actions in the clinic have been recently performed. These include the use of IGFBP-3 alone or in combination with IGF-I (trade name IPLEX, formerly SomatoKine) to treat patients with primary IGF-I deficiency (IGFD), GH insensitivity syndrome (GHIS, Laron syndrome), severe burning injuries, catabolic stress or even cancer. IGFD and GHIS are often caused by mutated GH receptors and lead to growth retardation, increased circulating GH levels, reduced circulating IGF-I, -II and IGFBP-3's levels, and hyperglycemia (Woods et al. 1997). Many of these symptoms can be ameliorated by IGF-I treatment, which normalizes the growth and metabolic functions (LeRoith *et al.* 2001). As mentioned earlier, IGFBP-3 circulates with IGF-I as a 150 kDa complex that include the ALS protein (Firth & Baxter 2002). The purpose of the complex is to prolong the half-life of IGF-I in serum from minutes to hours. IPLEX is more effective than IGF-I alone in increasing and stabilizing serum IGF-I levels in IGFD and GHIS patients and is especially useful for treating children with severe short stature as it stimulates muscle protein synthesis and connects the metabolic conditions without causing adverse side-effect such as IGF-I-induced hypoglycemia (Kemp & Thrailkill 2005). The ability of IPLEX to prevent muscle wasting during fasting conditions has also been studied in semi-starved rat. The result shows that plasma IGF-I level is 20% higher in rats treated with complex than IGF-I alone and almost recover to the same amino acid concentrations in the plasma as freely-

fed control rats which were significantly reduced in saline and IGF-I alone treatments (Svanberg *et al.* 2000). Furthermore, only IPLEX stimulated amino acid uptake, as assessed by radio-labeled phenylalanine incorporation, in muscle during long term starvation experiments (Svanberg et al. 2000), indicating that IGF-I alone is once again insufficient.

Another potential therapeutic use of IPLEX is for treating severely burned patients. Burn injuries induce cachexia, hypermetabolism, inflammation and immune system dysfunction (Herndon *et al.* 1999). Treating burn patients with GH and IGF-I can effectively enhance recovery of muscle wasting and increase resistance to infections (Edmondson *et al.* 2003). However, GH and IGF-I treatments normally lead to hyperglycemia (Singh *et al.* 1998) and hypoglycemia (Clark 2004), respectively. Administering IPLEX to severe burned adults (Debroy *et al.* 1999) and children (Herndon *et al.* 1999) successfully improved the net protein synthesis and maintained high serum IGF-I concentrations. In the adult patients, who had 24% total body surface area (TBSA) burns, serum IGF-I levels were raised from 118±11 to 489±90 ng/ml with 1.0 mg/kg/day IPLEX for five days (Debroy *et al.* 1999). In the children, who had >40 % TBSA burns and had received three times surgical procedures, serum IGF-I levels were raised from 100±10 to 275±52 μg/ml using an IPLEX dose of 0.5mg/kg/day over five to seven days (Spies *et al*. 2002). None of these participants developed hypoglycemia or electrolyte imbalance which can be caused by IGF-I therapy. IPLEX also attenuates the induction of acute phase proteins and proinflammatory cytokines after tissue injuries associated with infections, inflammations or burn injury (Moshage 1997). Proinflammatory cytokines aid in tissue recovery and help maintain systematic

homeostasis after trauma, however, overproduction can be detrimental and produce tissue damage itself, hypermetabolism, multiple organ failure or even death (Moshage 1997, Pruitt *et al.* 1995). Clinical studies indicate that IPLEX decreases the type I acute phase proteins and proinflammatory cytokines such as tumor necrosis factor (TNF) -α and interleukin-1β, both of which inhibit the GH/IGH-I growth axis and are associated with increased mortality in severely burned children (Jeschke *et al.* 2000, Pruitt *et al.* 1995, Spies *et al.* 2002). These studies strongly indicate that IPLEX is an effective therapeutic for treating burn injuries as it stimulates muscle growth and prevents trauma-induced muscle wasting, stimulates tissue recovery and blocks immune responses that interfere with these processes.

Diabetes is the most common metabolic disease and affects over 14 million people in the United States alone (National Center for Chronic Disease Prevention and Health Promotion, www.cdc.gov). Type I diabetes, or insulin-dependent diabetes mellitus (IDDM), is caused by the autoimmune attack of the insulin-secreting β-cells in the pancreas and usually presents during childhood or early adulthood. This type of diabetes accounts for only 10% of diabetic, who require insulin supplements to regulate glucose metabolism. On the other hand, patients with type II diabetes or non-insulin-dependent diabetes mellitus (NIDDM), have normal or elevated circulating insulin levels. This disease usually presents in middle-age and is associated with obesity, although it is also the primary endocrine disorder of children due to the prevalence of obesity in our youth (Berry *et al.* 2006). Livers of type II diabetics are unable to respond to insulin, which produces hyperglycemia and in turn, hyperinsulinaemia. The exact causes of type II diabetes are unknown but are believed to be due to an increased susceptibility to

environmental influences (Hattersley & Pearson 2006). IGF-I possesses about 6% of insulin's ability to produce hypoglycemia (Guler *et al.* 1987) and can ameliorate diabetic symptoms at high concentrations (Zenobi *et al.* 1992). It has also been suggested as a potential therapy for some diabetics as it can inhibit β cell apoptosis as well (Thrailkill 2000). IGF-I is rapidly cleared, however, suggesting that IPLEX could enhance the therapeutic potential of IGF-I in treating diabetes. For patients with type I diabetes, administrating IPLEX at a dose of 2mg/kg/day for two weeks reduced the insulin dose requirement by 49% (Clemmons *et al.* 2000). When given to type II diabetics, IPLEX reduced the insulin dose requirement by 54% (Clemmons *et al.* 2005). None of the side effects associated with IGF-I therapy occur in both type I and type II diabetics, which include edema, weight gain, tachycardia, dyspnea, myalgia and fatigue (Jabri *et al.* 1994, Clemmons *et al.* 2000) developed. These studies suggest that IPLEX can significantly regulate carbohydrate metabolism and increase the efficiency of insulin supplementation without producing adverse side effects. These studies are also the basis for Phase II clinical trials that will assess the long-term safety of treating diabetics with IPLEX (www.insmed.com).

Myotonic muscular dystrophy (MD), the most prevalent form of muscular dystrophy, is a dominantly inherited disease caused by the unstable repeated expansion of CTG nucleotides (Type I MD) in the 3' UTR region of the myotonic dystrophy protein kinase (*DMPK*) gene or CCTG (Type II MD) nucleotides in the intron of zinc finger protein 9 (*ZNF9*) gene. This disease mainly affects the function of smooth and skeletal muscle cells and commonly leads to myotonia (Machuca-Tzili *et al.* 2005). Although there is no direct evidence that IPLEX can decrease MD symptoms, it reduces muscle

protein degradation and preserves the number of muscle fibers during atrophy (Zdanowicz & Teichberg 2003). Administration of IGF-I (1mg/kg) for eight weeks to *mdx* mice- a murine model for Duchenne muscular dystrophy- increases the force per cross-sectional area of diaphragm by 49% and enhances fatigue resistance as well (Gregorevic et al. 2002). The growth promoting effects of IPLEX are greater and softer than IGF-I therapy and thus, IPLEX is a likely candidate therapeutic for treating some muscular dystrophies as well.

IGFBP-3 is a multifunctional molecule with potent anti-tumor properties. It regulates the tumor-promoting actions of IGF-I directly (Grimberg & Cohen 2000), but also inhibits tumor growth via IGF-independent means in several cancer cell types (Firth & Baxter 2002, Silha *et al*. 2006). Insmed is also conducting clinical trials (Phase I) on the growth inhibitory effects of IGFBP-3 in breast and colorectal cancer cells, a study that is based on several previous studies performed independently of Insmed (Butt *et al*. 2002, Gucev *et al*. 1996, LeRoith & Roberts 2003). Blouin *et al*. 2003 demonstrated that IGFBP-3 reduced the proliferation rate of MCF-7 breast cancer cells by 55% and HT-29 colorectal cancer cells by 49%. The co-administration of IGFBP-3 with 2 Gy radiation therapy has an additive effect on inhibiting the growth of MCF-7 and HT-29 carcinoma cells by 3.5 and 1.6 fold. The same study also investigated the *in vivo* effect of IGFBP-3. These receiving MCF-7 xenografts were injected twice daily with 20mg/kg IGFBP-3, only 3of 12 mice formed tumors with a mean volume 37.7mm^3 in the IGFBP-3 treated group while 8 of 12 control mice formed tumors with a mean volume 113.9mm^3 in control group. Thus, IGFBP-3 reduced both the incidence and size of MCF-7 tumors. IGFBP-3's anti-tumor functions are also enhanced when given in combination with

chemotherapeutic drugs (Yu *et al*. 2003). When using the same MCF-7 breast cancer tumor xenografts model, co-treatment of 10 mg/kg IGFBP-3 and 13 or 20 mg/kg Paclitaxel was 33% and 61%, respectively, more effective than Paclitaxel treatment alone. Similar results were also obtained with LoVo colorectal carcinoma xenografts, as treating with 30 mg/kg IGFBP-3 and 10 mg/kg CPT-11 was 30% to 69% more effective than CPT-11 alone. These studies suggest IGFBP-3 can be used as a not only single anti-tumor treatment, but that it also significantly enhances the efficiency of other chemotherapeutic therapies. However, the co-treatment of IGFBP-3 with either radiation or chemotherapeutic drugs may inhibit proliferation effects differently depending on the agents and on whether the cells are transformed or not (Perks *et al*. 2003). **Most importantly, the apoptotic functions of IGFBP-3 in cancer cells is largely IGF-independent (Lee & Cohen 2002) and often requires nuclear translocation (Firth *et al.* 2002).** These growth inhibitory functions need to be further characterized, however, as our observation that IGFBP-3 binds Rpb3 in the nucleus which is expressed ubiquitously in all cell types- suggests that IGFBP-3 may influence gene expression by directly modulating RNAPII activity in a tissue specific manner that depends on other interacting proteins. Nevertheless, its apoptotic actions in different cancer cells and its role in regulating IGF-I levels in circulation indicate that the potential clinical benefit of IGFBP-3 is not limited to fighting tumors, but is multifunctional.

Animal production implications

The IGF-I system has been implicated in many aspects of bovine mammary gland development and function (Akers 2006). As mentioned earlier, IGFBP-3 mediates the

inhibitory functions of TGF-β and retinoid acid on transformed mammary epithelial cells, which represses IGF-I stimulated proliferation (Gucev *et al.* 1996). However, IGFBP-3 possesses inverse functions in normal breast epithelial cells that are concentration dependent. It decreases cell number by 27.8% at lower concentrations (25 ng/ml) but increases cell number by 37 % at higher concentrations (100ng/ml) (Perks *et al.* 2002). The production of IGFBP-3 is primarily regulated by the GH/IGF-I axis. Therefore, these findings suggest that IGFBP-3 is an important regulator of breast epithelial cell proliferation and that it may help mediate GH-stimulated lactation efficiency.

The dual natural of IGFBP-3's actions also extend to the regulation of skeletal muscle growth and development. Although it supports growth by maintaining a circulating reservoir of IGF-I, it also mediates the suppressive effects of transforming growth factor (TGF)-β and myostatin on skeletal muscle cell lines and on porcine embryonic myogenic cells (Kamaga-Sollo *et al.* 2003). Myostatin is an extremely potent inhibitor of skeletal muscle growth as it directly inhibits myoblast proliferation. Mutations in the myostatin gene can create double-muscling in domestic livestock that similar to the myostatin null mouse (McPherron & Lee 1997). Thus, manipulating the production and/ or bioactivity of IGFBP-3 could potentially perturb myostatin's anti-proliferative actions in muscle cells and augment the muscle mass of domesticated species.

CHAPTER FOUR

REFERENCES

1. Adams GR 2002 Exercise Effects on Muscle Insulin Signaling and Action: Invited Review: Autocrine/paracrine IGF-I and skeletal muscle adaptation. J Appl Physiol 93:1159-1167
2. Akers RM 2006 Major Advances Associated with Hormone and Growth Factor Regulation of Mammary Growth and Lactation in Dairy Cows. J. Dairy Sci. 89:1222-1234
3. Angelloz-Nicoud P& Binoux M 1995 Autocrine regulation of cell proliferation by the insulin-like growth factor (IGF) and IGF binding protein-3 protease system in a human prostate carcinoma cell line (PC-3)136.12.5485. Endocrinology 136:5485-5492
4. Bach LA, Thotakura NR& Rechler MM 1992 Human Insulin-Like Growth-Factor Binding Protein-6 Is O-Glycosylated. Biochemical and Biophysical Research Communications 186:301-307
5. Backer JM, Myers MG, Jr., Shoelson SE, Chin DJ, Sun XJ, Miralpeix M, Hu P, Margolis B, Skolnik EY, Schlessinger J& et al. 1992 Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. Embo J 11:3469-3479
6. Berry D, Urban A& Grey M 2006 Understanding the Development and Prevention of Type 2 Diabetes in Youth (Part 1). Journal of Pediatric Health Care 20:3-10
7. Boyle BJ, Zhao XY, Cohen P& Feldman D 2001 Insulin-like growth factor binding protein-3 mediates 1 alpha,25-dihydroxyvitamin d(3) growth inhibition in the LNCaP prostate cancer cell line through p21/WAF1. J Urol 165:1319-1324
8. Buckway CK, Wilson EM, Ahlsen M, Bang P, Oh Y& Rosenfeld RG 2001 Mutation of three critical amino acids of the N-terminal domain of IGF-binding protein-3 essential for high affinity IGF binding. J Clin Endocrinol Metab 86:4943-4950
9. Butt AJ, Fraley KA, Firth SM& Baxter RC 2002 IGF-Binding Protein-3-Induced Growth Inhibition and Apoptosis Do Not Require Cell Surface Binding and Nuclear Translocation in Human Breast Cancer Cells. Endocrinology 143:2693-2699
10. Carr J, Grant P, Francis G, Owens J, Wallace J& Walton P 1994 Isolation and characterization of ovine IGFBP-4: protein purification and cDNA sequence. J Mol Endocrinol 13:219-236
11. Cheung CW, Vesey DA, Nicol DL& Johnson DW 2004 The roles of IGF-I and IGFBP-3 in the regulation of proximal tubule, and renal cell carcinoma cell proliferation. Kidney Int 65:1272-1279
12. Clark RG 2004 Recombinant human insulin-like growth factor I (IGF-I): risks and benefits of normalizing blood IGF-I concentrations. Horm Res 62 Suppl 1:93-100
13. Clemmons DR, Moses AC, McKay MJ, Sommer A, Rosen DM& Ruckle J 2000 The Combination of Insulin-Like Growth Factor I and Insulin-Like Growth Factor-Binding Protein-3 Reduces Insulin Requirements in Insulin-Dependent

Type 1 Diabetes: Evidence for in VivoBiological Activity. J Clin Endocrinol Metab 85:1518-1524
14. Clemmons DR 2001 Use of Mutagenesis to Probe IGF-Binding Protein Structure/Function Relationships. Endocr Rev 22:800-817
15. Clemmons DR, Moses AC, Sommer A, Jacobson W, Rogol AD, Sleevi MR& Allan G 2005 Rh/IGF-I/rhIGFBP-3 administration to patients with type 2 diabetes mellitus reduces insulin requirements while also lowering fasting glucose. Growth Hormone & IGF Research 15:265-274
16. Cohen P, Lamson G, Okajima T& Rosenfeld RG 1993 Transfection of the human insulin-like growth factor binding protein-3 gene into Balb/c fibroblasts inhibits cellular growth. Mol Endocrinol 7:380-386
17. Cohen P, Peehl DM, Graves HC& Rosenfeld RG 1994 Biological effects of prostate specific antigen as an insulin-like growth factor binding protein-3 protease. J Endocrinol 142:407-415
18. Cohen P, Nunn SE& Peehl DM 2000 Transforming growth factor-beta induces growth inhibition and IGF-binding protein-3 production in prostatic stromal cells: abnormalities in cells cultured from benign prostatic hyperplasia tissues. J Endocrinol 164:215-223
19. Conover CA, Clarkson JT& Bale LK 1996 Factors regulating insulin-like growth factor-binding protein-3 binding, processing, and potentiation of insulin-like growth factor action. Endocrinology 137:2286-2292
20. Corbi N, Di Padova M, De Angelis R, Bruno T, Libri V, Iezzi S, Floridi A, Fanciulli M& Passananti C 2002 The a-like RNA polymerase II core subunit 3 (RPB3) is involved in tissue-specific transcription and muscle differentiation via interaction with the myogenic factor myogenin. FASEB J.:02-0123fje
21. Cramer P 2004 RNA polymerase II structure: from core to functional complexes. Current Opinion in Genetics & Development 14:218-226
22. De Angelis R, Iezzi S, Bruno T, Corbi N, Di Padova M, Floridi A, Fanciulli M& Passananti C 2003 Functional interaction of the subunit 3 of RNA polymerase II (RPB3) with transcription factor-4 (ATF4). FEBS Letters 547:15-19
23. De Mellow JSM& Baxter RC 1988 Growth hormone-dependent insulin-like growth factor (IGF) binding protein both inhibits and potentiates IGF-I-stimulated DNA synthesis in human skin fibroblasts. Biochemical and Biophysical Research Communications 156:199-204
24. Debroy MA, Wolf SE, Zhang XJ, Chinkes DL, Ferrando AA, Wolfe RR& Herndon DN 1999 Anabolic effects of insulin-like growth factor in combination with insulin-like growth factor binding protein-3 in severely burned adults. J Trauma 47:904-910; discussion 910-901
25. Duan C& Xu Q 2005 Roles of insulin-like growth factor (IGF) binding proteins in regulating IGF actions. General and Comparative Endocrinology 142:44-52
26. Edmondson SR, Thumiger SP, Werther GA& Wraight CJ 2003 Epidermal homeostasis: the role of the growth hormone and insulin-like growth factor systems. Endocr Rev 24:737-764
27. Fanayan S, Firth SM, Butt AJ& Baxter RC 2000 Growth inhibition by insulin-like growth factor-binding protein-3 in T47D breast cancer cells requires transforming

growth factor-beta (TGF-beta) and the type II TGF-beta receptor. J Biol Chem 275:39146-39151
28. Fanciulli M, Bruno T, Cerboni C, Bonetto F, Iacobini C, Frati L, Piccoli M, Floridi A, Santoni A& Punturieri A 1996 Cloning of a novel human RNA polymerase II subunit downregulated by doxorubicin: new potential mechanisms of drug related toxicity. FEBS Letters 384:48-52
29. Fanciulli M, Bruno T, Di Padova M, De Angelis R, Lovari S, Floridi A& Passananti C 1998 The interacting RNA polymerase II subunits, hRPB11 and hRPB3, are coordinately expressed in adult human tissues and down-regulated by doxorubicin. FEBS Letters 427:236-240
30. Firth S& Baxter R 1999 Characterisation of recombinant glycosylation variants of insulin-like growth factor binding protein-3. J Endocrinol 160:379-387
31. Firth SM& Baxter RC 2002 Cellular Actions of the Insulin-Like Growth Factor Binding Proteins. Endocr Rev 23:824-854
32. Florini J, Ewton D& Coolican S 1996 Growth hormone and the insulin-like growth factor system in myogenesis. Endocr Rev 17:481-517
33. Foulstone EJ, Savage PB, Crown AL, Holly JM& Stewart CE 2003 Role of insulin-like growth factor binding protein-3 (IGFBP-3) in the differentiation of primary human adult skeletal myoblasts. J Cell Physiol 195:70-79
34. Gregorevic P, Plant DR, Leeding KS, Bach LA& Lynch GS 2002 Improved contractile function of the mdx dystrophic mouse diaphragm muscle after insulin-like growth factor-I administration. Am J Pathol 161:2263-2272
35. Grimberg A& Cohen P 2000 Role of insulin-like growth factors and their binding proteins in growth control and carcinogenesis. J Cell Physiol 183:1-9
36. Gucev ZS, Oh Y, Kelley KM& Rosenfeld RG 1996 Insulin-like growth factor binding protein 3 mediates retinoic acid- and transforming growth factor beta2-induced growth inhibition in human breast cancer cells. Cancer Res 56:1545-1550
37. Guler H, Zapf J& Froesch E 1987 Short-term metabolic effects of recombinant human insulin-like growth factor I in healthy adults. N Engl J Med 317:137-140
38. Hampel B, Wagner M, Teis D, Zwerschke W, Huber LA& Jansen-Durr P 2005 Apoptosis resistance of senescent human fibroblasts is correlated with the absence of nuclear IGFBP-3. Aging Cell 4:325-330
39. Hattersley AT& Pearson ER 2006 Minireview: Pharmacogenetics and Beyond: The Interaction of Therapeutic Response, {beta}-Cell Physiology, and Genetics in Diabetes. Endocrinology 147:2657-2663
40. Herndon DN, Ramzy PI, DebRoy MA, Zheng M, Ferrando AA, Chinkes DL, Barret JP, Wolfe RR& Wolf SE 1999 Muscle protein catabolism after severe burn: effects of IGF-1/IGFBP-3 treatment. Ann Surg 229:713-720; discussion 720-712
41. Hoeck WG& Mukku VR 1994 Identification of the Major Sites of Phosphorylation in Igf Binding Protein-3. Journal of Cellular Biochemistry 56:262-273
42. Hollowood AD, Stewart CEH, Perks CM, Pell JM, Lai T, Alderson D& Holly JMP 2002 Evidence implicating a mid-region sequence of IGFBP-3 in its specific IGF-independent actions. Journal of Cellular Biochemistry 86:583-589

43. Hong J, Zhang G, Dong F& Rechler MM 2002 Insulin-like growth factor (IGF)-binding protein-3 mutants that do not bind IGF-I or IGF-II stimulate apoptosis in human prostate cancer cells. J Biol Chem 277:10489-10497
44. Jabri N, Schalch DS, Schwartz SL, Fischer JS, Kipnes MS, Radnik BJ, Turman NJ, Marcsisin VS& Guler HP 1994 Adverse effects of recombinant human insulin-like growth factor I in obese insulin-resistant type II diabetic patients. Diabetes 43:369-374
45. Jaques G, Noll K, Wegmann B, Witten S, Kogan E, Radulescu RT& Havemann K 1997 Nuclear Localization of Insulin-Like Growth Factor Binding Protein 3 in a Lung Cancer Cell Line. Endocrinology 138:1767-
46. Jeschke MG, Barrow RE& Herndon DN 2000 Insulinlike growth factor I plus insulinlike growth factor binding protein 3 attenuates the proinflammatory acute phase response in severely burned children. Ann Surg 231:246-252
47. Johnson BJ, White ME, Hathaway MR& Dayton WR 1999 Decreased steady-state insulin-like growth factor binding protein-3 (IGFBP-3) mRNA level is associated with differentiation of cultured porcine myogenic cells. J Cell Physiol 179:237-243
48. Johnson BJ, White ME, Hathaway MR& Dayton WR 2003 Effect of differentiation on levels of insulin-like growth factor binding protein mRNAs in cultured porcine embryonic myogenic cells. Domestic Animal Endocrinology 24:81-93
49. Jones JI, D'Ercole AJ, Camacho-Hubner C& Clemmons DR 1991 Phosphorylation of insulin-like growth factor (IGF)-binding protein 1 in cell culture and in vivo: effects on affinity for IGF-I. Proceedings Of The National Academy Of Sciences Of The United States Of America 88:7481-7485
50. Kamanga-Sollo E, Pampusch MS, White ME& Dayton WR 2003 Role of insulin-like growth factor binding protein (IGFBP)-3 in TGF-beta- and GDF-8 (myostatin)-induced suppression of proliferation in porcine embryonic myogenic cell cultures. J Cell Physiol 197:225-231
51. Kamanga-Sollo E, Pampusch MS, White ME, Hathaway MR& Dayton WR 2005 Insulin-like growth factor binding protein (IGFBP)-3 and IGFBP-5 mediate TGF-beta- and myostatin-induced suppression of proliferation in porcine embryonic myogenic cell cultures. Exp Cell Res 311:167-176
52. Kemp SF& Thrailkill KM 2005 SomatoKine: is there a use in treating growth disorders? Curr Opin Investig Drugs 6:373-377
53. Kim HS, Ingermann AR, Tsubaki J, Twigg SM, Walker GE& Oh Y 2004 Insulin-like growth factor-binding protein 3 induces caspase-dependent apoptosis through a death receptor-mediated pathway in MCF-7 human breast cancer cells. Cancer Res 64:2229-2237
54. Lalou C, Lassarre C& Binoux M 1995 A proteolytic fragment of insulin-like growth factor (IGF) binding protein-3 that fails to bind IGF is a cell growth inhibitor. Prog Growth Factor Res 6:311-316
55. Lalou C, Lassarre C& Binoux M 1996 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. Endocrinology 137:3206-3212

56. Lawlor MA& Rotwein P 2000 Insulin-Like Growth Factor-Mediated Muscle Cell Survival: Central Roles for Akt and Cyclin-Dependent Kinase Inhibitor p21. Mol. Cell. Biol. 20:8983-8995
57. Le Roith D, Scavo L& Butler A 2001 What is the role of circulating IGF-I? Trends Endocrinol Metab 12:48-52
58. Lee KW& Cohen P 2002 Nuclear effects: unexpected intracellular actions of insulin-like growth factor binding protein-3. J Endocrinol 175:33-40
59. Lee KW, Liu B, Ma L, Li H, Bang P, Koeffler HP& Cohen P 2004 Cellular internalization of insulin-like growth factor binding protein-3: distinct endocytic pathways facilitate re-uptake and nuclear localization. J Biol Chem 279:469-476
60. Lee KW, Ma L, Yan X, Liu B, Zhang XK& Cohen P 2005 Rapid apoptosis induction by IGFBP-3 involves an insulin-like growth factor-independent nucleomitochondrial translocation of RXRalpha/Nur77. J Biol Chem 280:16942-16948
61. LeRoith D, Werner H, Beitner-Johnson D& Roberts C, Jr 1995 Molecular and cellular aspects of the insulin-like growth factor I receptor. Endocr Rev 16:143-163
62. LeRoith D& Roberts CT, Jr. 2003 The insulin-like growth factor system and cancer. Cancer Lett 195:127-137
63. Li W, Fawcett J, Widmer HR, Fielder PJ, Rabkin R& Keller GA 1997 Nuclear transport of insulin-like growth factor-I and insulin-like growth factor binding protein-3 in opossum kidney cells. Endocrinology 138:1763-1766
64. Liu JP, Baker J, Perkins AS, Robertson EJ& Efstratiadis A 1993 Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell 75:59-72
65. Liu B, Lee HY, Weinzimer SA, Powell DR, Clifford JL, Kurie JM& Cohen P 2000 Direct functional interactions between insulin-like growth factor-binding protein-3 and retinoid X receptor-alpha regulate transcriptional signaling and apoptosis. J Biol Chem 275:33607-33613
66. Longobardi L, Torello M, Buckway C, O'Rear L, Horton WA, Hwa V, Roberts CT, Jr., Chiarelli F, Rosenfeld RG& Spagnoli A 2003 A novel insulin-like growth factor (IGF)-independent role for IGF binding protein-3 in mesenchymal chondroprogenitor cell apoptosis. Endocrinology 144:1695-1702
67. Ma J, Pollak MN, Giovannucci E, Chan JM, Tao Y, Hennekens CH& Stampfer MJ 1999 Prospective Study of Colorectal Cancer Risk in Men and Plasma Levels of Insulin-Like Growth Factor (IGF)-I and IGF-Binding Protein-3. J Natl Cancer Inst 91:620-625
68. Machuca-Tzili L, Brook D& Hilton-Jones D 2005 Clinical and molecular aspects of the myotonic dystrophies: a review. Muscle Nerve 32:1-18
69. Marinaro JA, Neumann GM, Russo VC, Leeding KS& Bach LA 2000 O-glycosylation of insulin-like growth factor (IGF) binding protein-6 maintains high IGF-II binding affinity by decreasing binding to glycosaminoglycans and susceptibility to proteolysis. European Journal Of Biochemistry / FEBS 267:5378-5386
70. Marshall CJ 1995 Specificity of receptor tyrosine kinase signaling: Transient versus sustained extracellular signal-regulated kinase activation. Cell 80:179-185

71. Mathews LS, Hammer RE, Behringer RR, D'Ercole AJ, Bell GI, Brinster RL& Palmiter RD 1988 Growth enhancement of transgenic mice expressing human insulin-like growth factor I. Endocrinology 123:2827-2833
72. Matsumoto T, Tsurumoto T, Goldring M& Shindo H 2000 Differential effects of IGF-binding proteins, IGFBP-3 and IGFBP-5, on IGF-I action and binding to cell membranes of immortalized human chondrocytes. J Endocrinol 166:29-37
73. McCaig C, Fowler CA, Laurence NJ, Lai T, Savage PB, Holly JM& Perks CM 2002 Differential interactions between IGFBP-3 and transforming growth factor-beta (TGF-beta) in normal vs cancerous breast epithelial cells. Br J Cancer 86:1963-1969
74. McPherron AC& Lee SJ 1997 Double muscling in cattle due to mutations in the myostatin gene. Proc Natl Acad Sci U S A 94:12457-12461
75. Moshage H 1997 Cytokines and the hepatic acute phase response. J Pathol 181:257-266
76. Oh Y, Muller HL, Ng L& Rosenfeld RG 1995 Transforming growth factor-beta-induced cell growth inhibition in human breast cancer cells is mediated through insulin-like growth factor-binding protein-3 action. J Biol Chem 270:13589-13592
77. Pampusch M, Kamanga-Sollo E, White M, Hathaway M& Dayton W 2003 Effect of recombinant porcine IGF-binding protein-3 on proliferation of embryonic porcine myogenic cell cultures in the presence and absence of IGF-I. J Endocrinol 176:227-235
78. Payet LD, Firth SM& Baxter RC 2004 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 89:2382-2389
79. Perks CM, McCaig C, Clarke JB, Clemmons DR& Holly JMP 2002 A non-IGF binding mutant of IGFBP-3 modulates cell function in breast epithelial cells. Biochemical and Biophysical Research Communications 294:988-994
80. Pruitt JH, Copeland EM, 3rd& Moldawer LL 1995 Interleukin-1 and interleukin-1 antagonism in sepsis, systemic inflammatory response syndrome, and septic shock. Shock 3:235-251
81. Rajah R, Valentinis B& Cohen P 1997 Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor-beta1 on programmed cell death through a p53- and IGF-independent mechanism. J Biol Chem 272:12181-12188
82. Rozen F, Zhang J& Pollak M 1998 Antiproliferative action of tumor necrosis factor-alpha on MCF-7 breastcancer cells is associated with increased insulin-like growth factor binding protein-3 accumulation. Int J Oncol 13:865-869
83. Schedlich LJ, Young TF, Firth SM& Baxter RC 1998 Insulin-like Growth Factor-binding Protein (IGFBP)-3 and IGFBP-5 Share a Common Nuclear Transport Pathway in T47D Human Breast Carcinoma Cells. J. Biol. Chem. 273:18347-18352
84. Schedlich LJ, Le Page SL, Firth SM, Briggs LJ, Jans DA& Baxter RC 2000 Nuclear import of insulin-like growth factor-binding protein-3 and -5 is mediated by the importin beta subunit. J Biol Chem 275:23462-23470

85. Schedlich LJ& Graham LD 2002 Role of insulin-like growth factor binding protein-3 in breast cancer cell growth. Microsc Res Tech 59:12-22
86. Scherer PE, Lewis RY, Volonte D, Engelman JA, Galbiati F, Couet J, Kohtz DS, van Donselaar E, Peters P& Lisanti MP 1997 Cell-type and Tissue-specific Expression of Caveolin-2. CAVEOLINS 1 AND 2 CO-LOCALIZE AND FORM A STABLE HETERO-OLIGOMERIC COMPLEX IN VIVO. J. Biol. Chem. 272:29337-29346
87. Schmid C, Ghirlanda-Keller C& Zapf J 2001 Effects of IGF-I and -II, IGF binding protein-3 (IGFBP-3), and transforming growth factor-beta (TGF-beta) on growth and apoptosis of human osteosarcoma Saos-2/B-10 cells: lack of IGF-independent IGFBP-3 effects. Eur J Endocrinol 145:213-221
88. Shimo-Oka T, Hagiwara Y& Ozawa E 1986 Class specificity of transferrin as a muscle trophic factor. J Cell Physiol 126:341-351
89. Silha JV, Sheppard PC, Mishra S, Gui Y, Schwartz J, Dodd JG& Murphy LJ 2006 Insulin-like growth factor (IGF) binding protein-3 attenuates prostate tumor growth by IGF-dependent and IGF-independent mechanisms. Endocrinology 147:2112-2121
90. Singh KP, Prasad R, Chari PS& Dash RJ 1998 Effect of growth hormone therapy in burn patients on conservative treatment. Burns 24:733-738
91. Singh B, Charkowicz D& Mascarenhas D 2004 Insulin-like growth factor-independent effects mediated by a C-terminal metal-binding domain of insulin-like growth factor binding protein-3. J Biol Chem 279:477-487
92. Spies M, Wolf SE, Barrow RE, Jeschke MG& Herndon DN 2002 Modulation of types I and II acute phase reactants with insulin-like growth factor-1/binding protein-3 complex in severely burned children. Crit Care Med 30:83-88
93. Svanberg E, Ohlsson C, Kimball SR& Lundholm K 2000 rhIGF-I/IGFBP-3 complex, but not free rhIGF-I, supports muscle protein biosynthesis in rats during semistarvation. Eur J Clin Invest 30:438-446
94. Tan Q, Linask KL, Ebright RH& Woychik NA 2000 Activation mutants in yeast RNA polymerase II subunit RPB3 provide evidence for a structurally conserved surface required for activation in eukaryotes and bacteria. Genes Dev. 14:339-348
95. Thrailkill KM 2000 Insulin-like growth factor-I in diabetes mellitus: its physiology, metabolic effects, and potential clinical utility. Diabetes Technol Ther 2:69-80
96. Tomas FM, Knowles SE, Owens PC, Chandler CS, Francis GL, Read LC& Ballard FJ 1992 Insulin-like growth factor-I (IGF-I) and especially IGF-I variants are anabolic in dexamethasone-treated rats. Biochem J 282 (Pt 1):91-97
97. Woods KA, Dastot F, Preece MA, Clark AJL, Postel-Vinay M-C, Chatelain PG, Ranke MB, Rosenfeld RG, Amselem S& Savage MO 1997 Phenotype: Genotype Relationships in Growth Hormone Insensitivity Syndrome. J Clin Endocrinol Metab 82:3529-3535
98. Wraight CJ, Liepe IJ, White PJ, Hibbs AR& Werther GA 1998 Intranuclear localization of insulin-like growth factor binding protein-3 (IGFBP-3) during cell division in human keratinocytes. J Invest Dermatol 111:239-242
99. Xi G, Kamanga-Sollo E, Pampusch MS, White ME, Hathaway MR& Dayton WR 2004 Effect of recombinant porcine IGFBP-3 on IGF-I and long-R3-IGF-I-

stimulated proliferation and differentiation of L6 myogenic cells. J Cell Physiol 200:387-394
100. Xiao S, Rose D, Sasaoka T, Maegawa H, Burke T, Jr, Roller P, Shoelson S& Olefsky J 1994 Syp (SH-PTP2) is a positive mediator of growth factor-stimulated mitogenic signal transduction. J. Biol. Chem. 269:21244-21248
101. Yamamoto K, Altschuler D, Wood E, Horlick K, Jacobs S& Lapetina E 1992 Association of phosphorylated insulin-like growth factor-I receptor with the SH2 domains of phosphatidylinositol 3-kinase p85. J. Biol. Chem. 267:11337-11343
102. Yan X, Forbes BE, McNeil KA, Baxter RC& Firth SM 2004 Role of N- and C-terminal residues of insulin-like growth factor (IGF)-binding protein-3 in regulating IGF complex formation and receptor activation. J Biol Chem 279:53232-53240
103. Yu H, Spitz MR, Mistry J, Gu J, Hong WK& Wu X 1999 Plasma levels of insulin-like growth factor-I and lung cancer risk: a case-control analysis. J Natl Cancer Inst 91:151-156
104. Zdanowicz MM& Teichberg S 2003 Effects of Insulin-Like Growth Factor-1/Binding Protein-3 Complex on Muscle Atrophy in Rats. Experimental Biology and Medicine 228:891-897
105. Zenobi PD, Jaeggi-Groisman SE, Riesen WF, Roder ME& Froesch ER 1992 Insulin-like growth factor-I improves glucose and lipid metabolism in type 2 diabetes mellitus. J Clin Invest 90:2234-2241