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

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Abstract

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

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

Introduction

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

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

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

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

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

Materials and methods

Cell lines and materials

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

ELISA

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

RNA collection and Quantitative real-time PCR

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

Luciferase constructs and Sp1 plasmids

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

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

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

Cell lysates and nuclear/cytoplasmic fractionation

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

Transfections, western blots, and luciferase measurements

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

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

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

Immunofluorescence

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

Immunoprecipitation and chromatin immunoprecipitation

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

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

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

Results

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

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

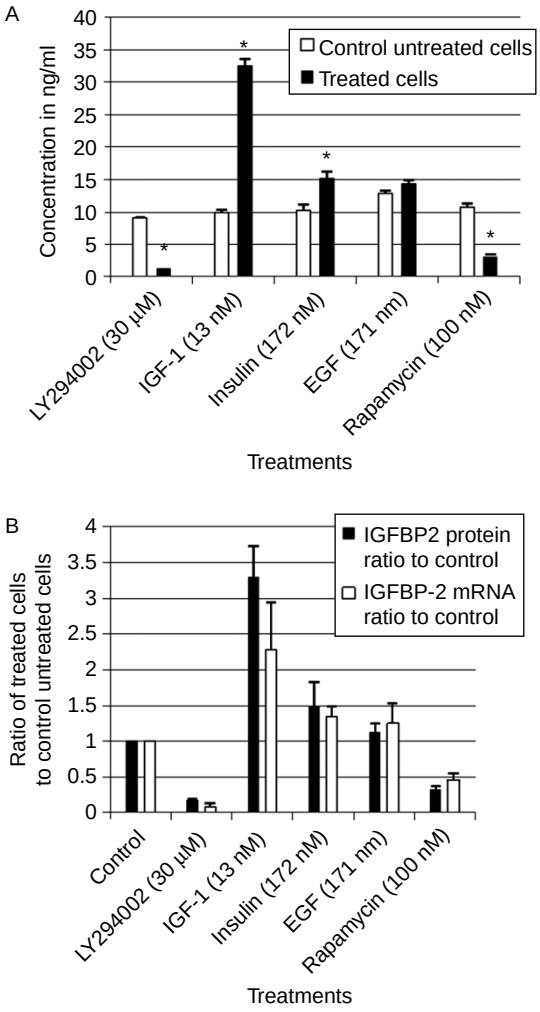

Figure 1. Effect of PI3K/AKT/mTOR pathway modulators on IGBP-2 protein and mRNA expression in MCF-7 breast cancer cells. (A) Concentration of IGFBP-2 protein in conditioned media of MCF-7 cells. Cells were plated at 5×10^5 cells per well in six-well plates and cultured for 24 h. The media were then exchanged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

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

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

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

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

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

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

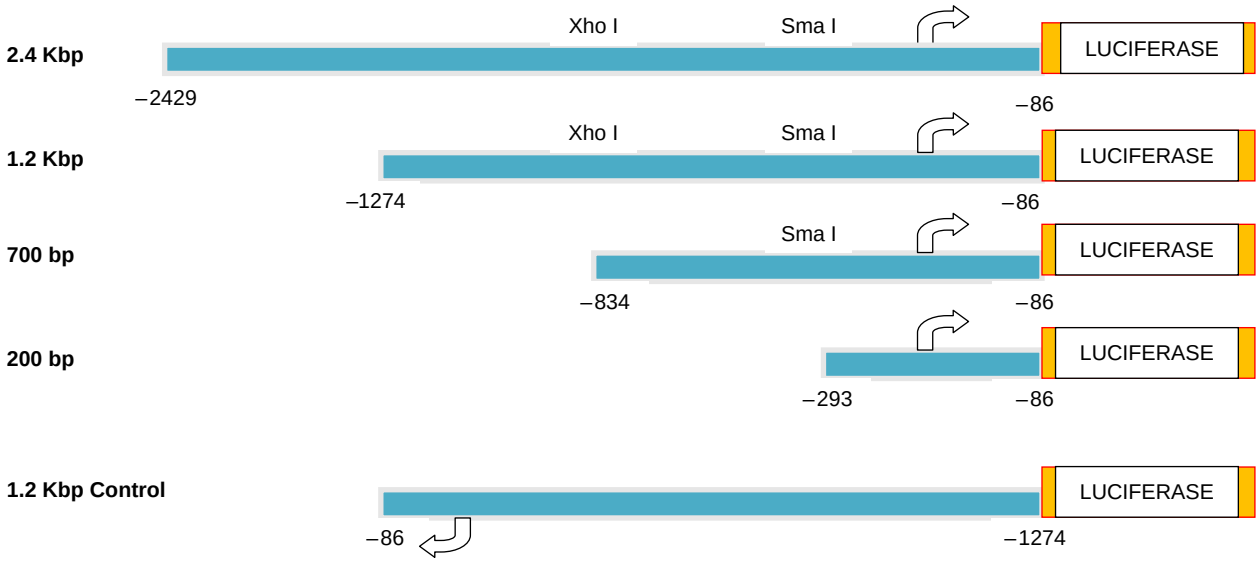

Figure 2. Schematic diagram of luciferase reporter assay constructs. A 2.4 and 1.2 kbp sequence upstream of the translation initiation site were cloned into the PGL-3 vector. Positions relative to the first ATG of IGFBP-2 are indicated below, the arrow represents the IGFBP-2 transcription initiation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

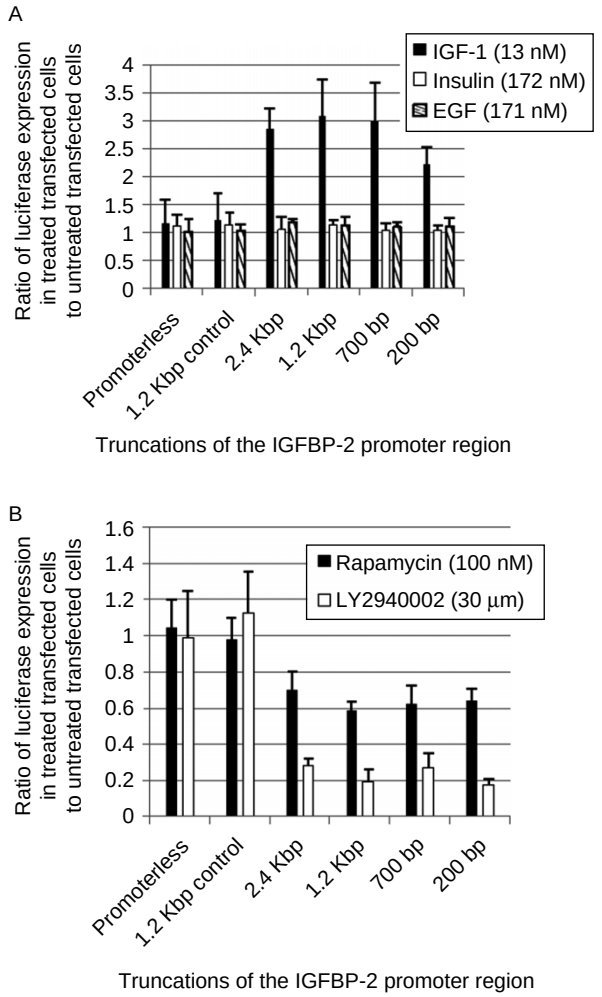

Figure 3. Effect of PI3K/AKT/mTOR pathway modulators on serially truncated IGFBP-2 promoter region sequences. (A) Effect of activators of the pathway. 4×10^5 MCF-7 cells were transfected with 2 μg of DNA per well in 24-well plates using electroporation. Cells were cultured for 24 h and then transferred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

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

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

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

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

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

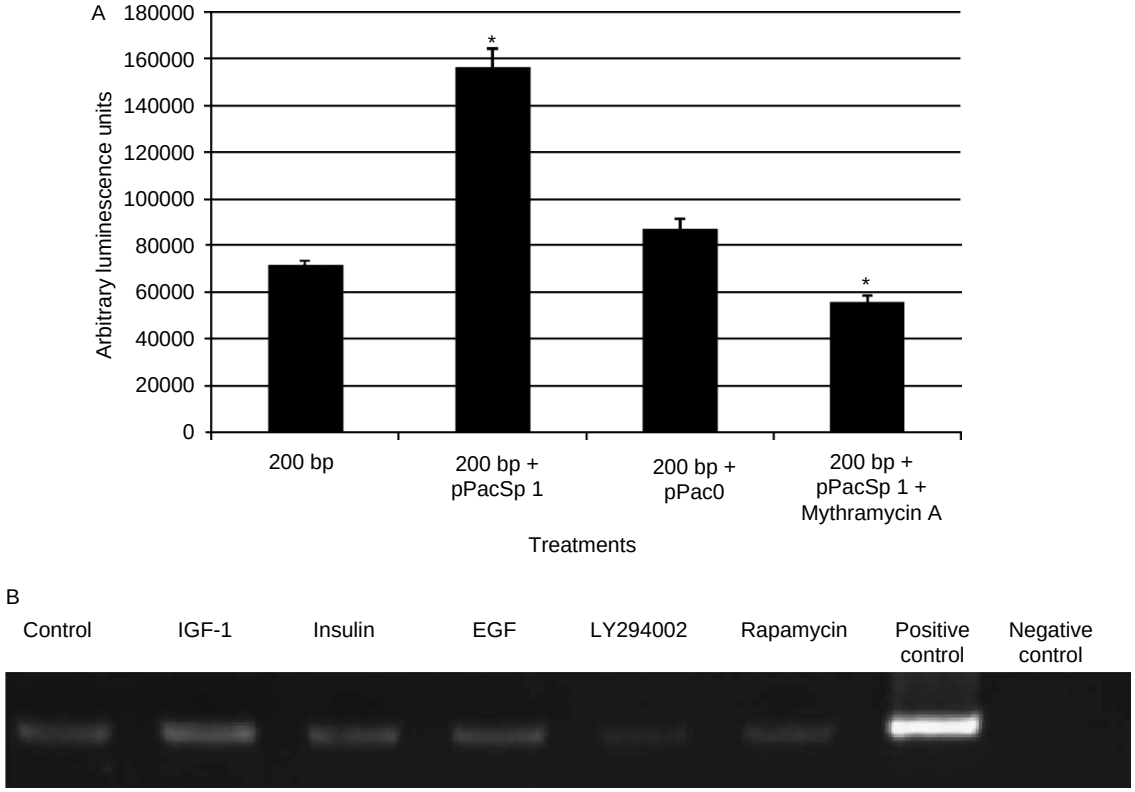

Figure 4. Effects of Sp1 on IGFBP-2 promoter transactivation. (A) The effect of overexpression of Sp1 on the 200 bp luciferase reporter construct. 4×10^5 cells were transfected with 2 μg of 200 bp construct or cotransfected with the former and 2 μg of pPac Sp1 or pPac 0 vector (Addgene) accordingly.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

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

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

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

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

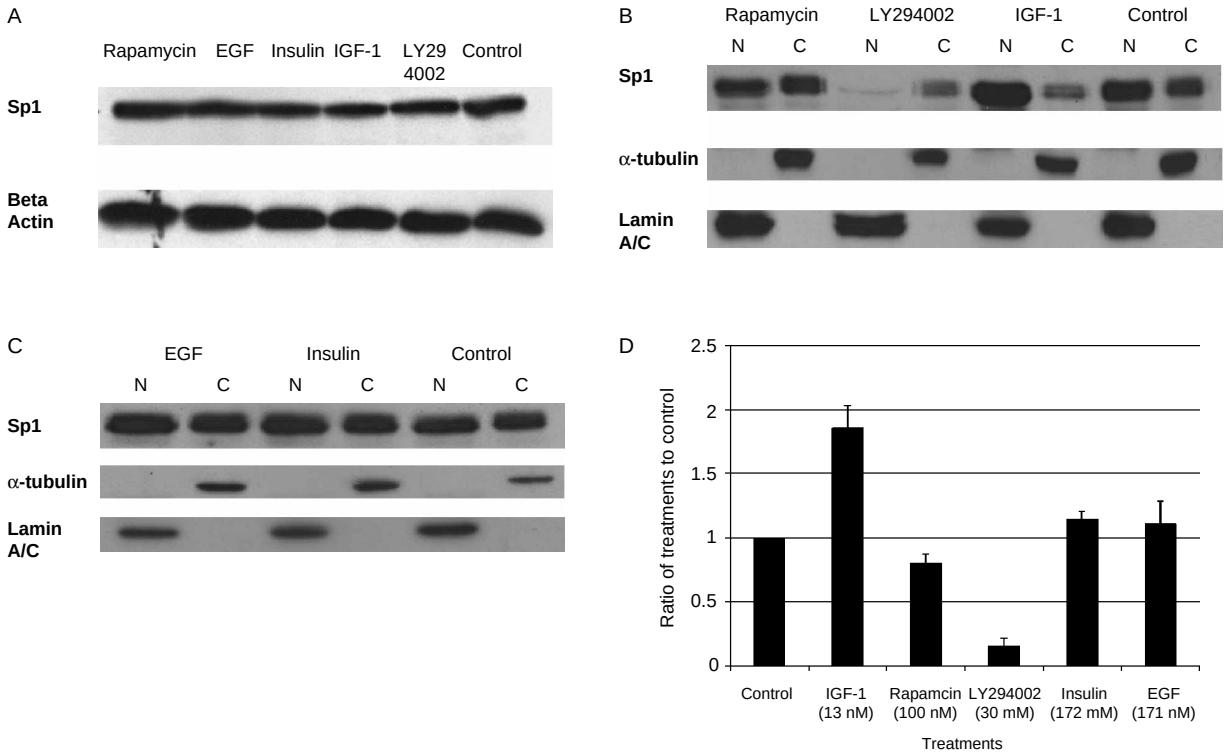

Figure 5. Effect of PI3K/AKT/mTOR pathway modulators on Sp1 protein nuclear and cytoplasmic abundance. (A) Effect on total Sp1 protein. Cells were cultured as described in previous figures and treated with appropriate growth factors (IGF-1 at 13 nM, insulin at 172 nM, EGF at 171 nM, LY294002 at 30 μM,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

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

Discussion

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

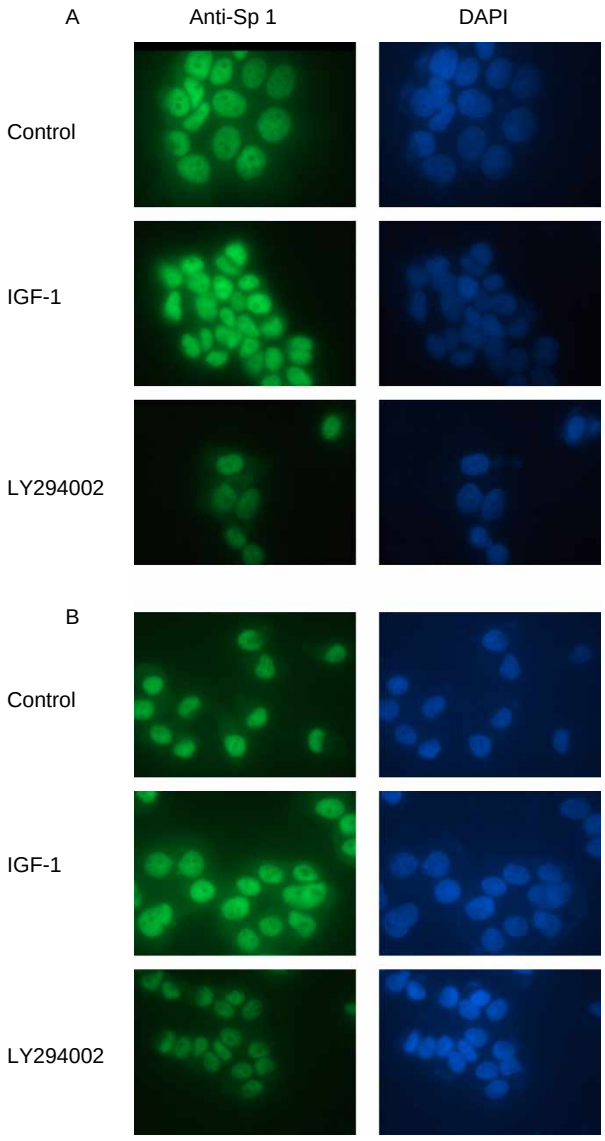

Figure 6. Immunofluorescence staining of Sp1. (A) MCF-7 cells were seeded at 5×10^4 cells per well in a Labtech eight-well microscopy slide and left to incubate for 24 h. Then, the media were changed to serum-free with the appropriate treatments [IGF-1 (13 nM) or LY294002 (30 μM)] for 72 h before immunostaining.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

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

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

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

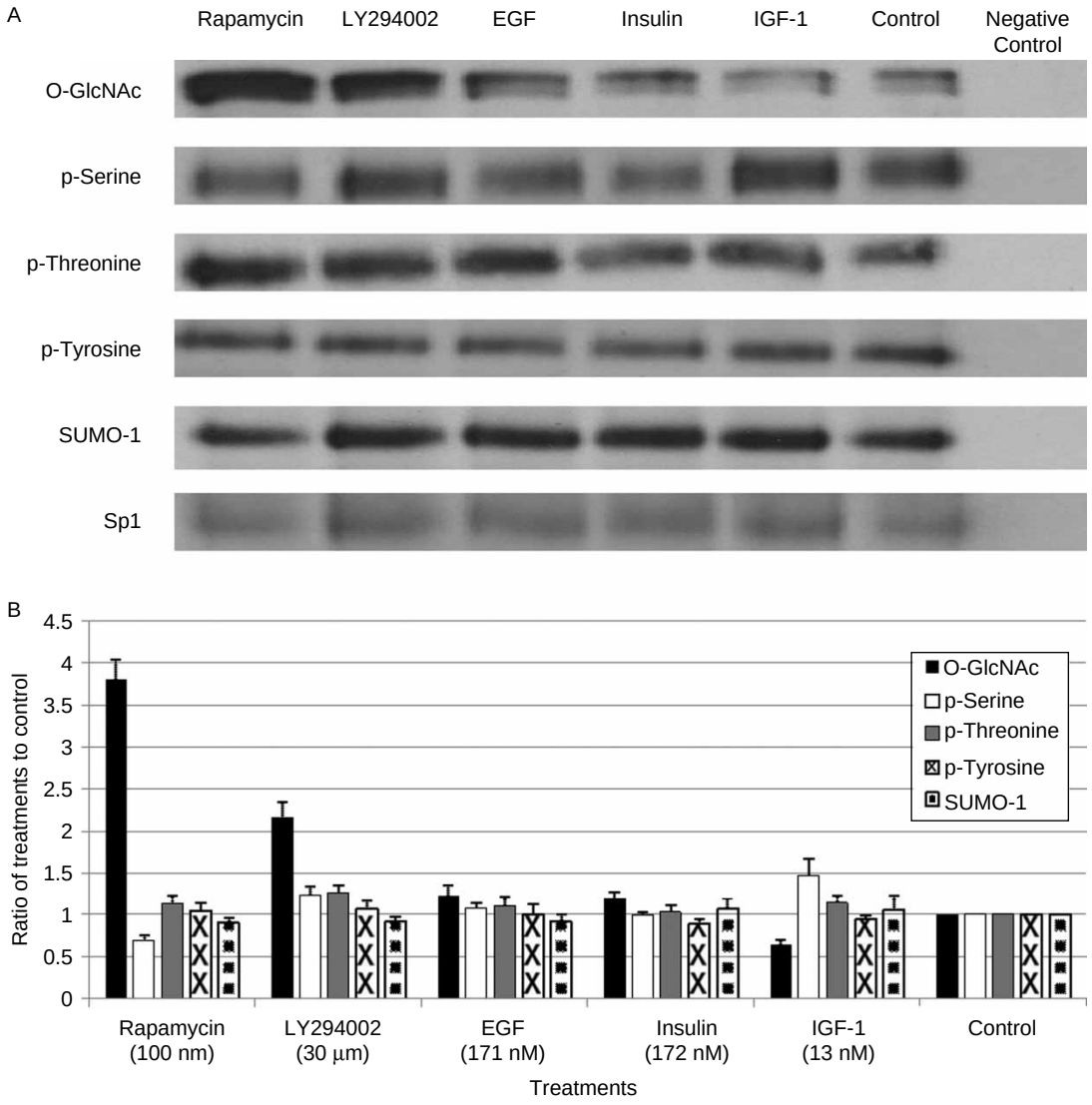

Figure 7. Effect of PI3K/AKT/mTOR pathway modulators on Sp1 posttranslational modifications. (A) Cells were cultured in 10% FBS media in 10 mm dishes until 70% confluence was reached, then treated for 72 h in serum-free media containing the appropriate compounds. Cells were then lyzed in RIPA buffer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

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

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

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

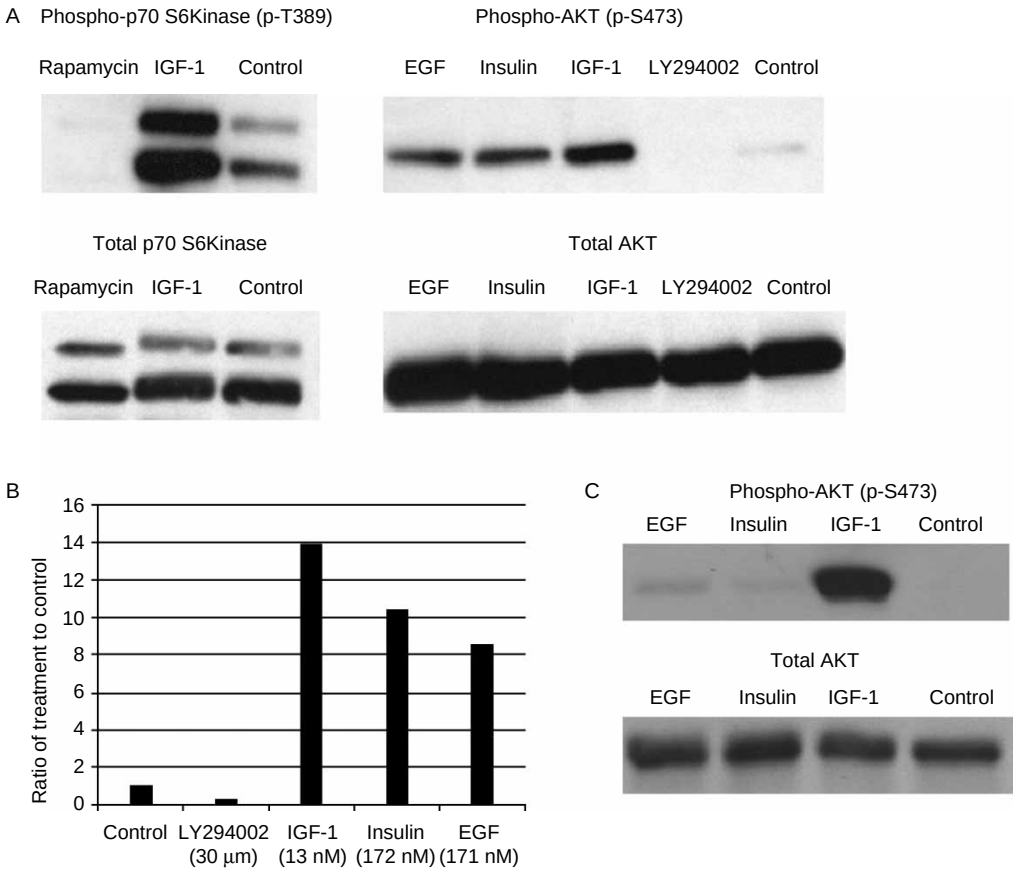

Figure 8. Signaling of PI3K/AKT/mTOR modulators. (A) Cells were seeded at 5×10^5 cells per well in six-well plates, left to incubate for 24 h, and transferred to serum-free conditions for an additional 24 h. Media were replaced with appropriate IGF-1 (13 nM), EGF (171 nM), insulin (172 nM) containing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

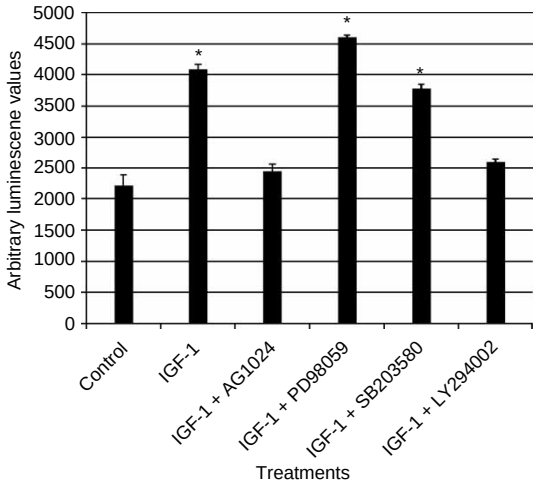

Figure 9. IGF-1 activation of the 200 bp construct. 4×10^5 cells were transfected with 2 μg of 200 bp construct. Twenty-four hours later, media were changed to serum-free control or IGF-1 (13 nM) or IGF-1 and the following AG1024 (10 μM), LY294002 (30 μM), PD98059 (30 μM), and SB203580 (10 μM). Seventy-two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

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

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