BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

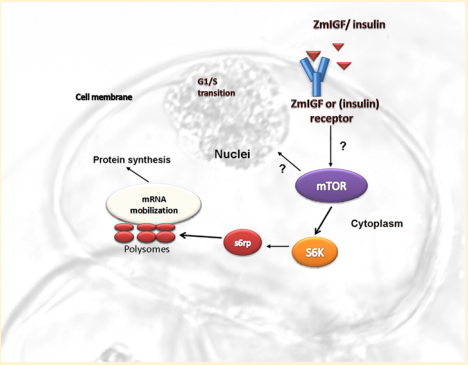
Insights into the TOR-S6K Signaling Pathway in Maize (*Zea mays* L.). Pathway Activation by Effector−Receptor Interaction

Verónica Garrocho-Villegas, Raúl Aguilar C, and Estela Sánchez de Jiménez*

Biochemistry Department, Chemistry Faculty, UNAM, Mexico City 04510, Mexico

Supporting Information

ABSTRACT: The primordial TOR pathway, known to control growth and cell proliferation, has still not been fully described for plants. Nevertheless, in maize, an insulin-like growth factor (ZmIGF) peptide has been reported to stimulate this pathway. This research provides further insight into the TOR pathway in maize, using a biochemical approach in cultures of fast-growing (FG) and slow-growing (SG) calli, as a model system. Our results revealed that addition of either ZmIGF or insulin to SG calli stimulated DNA synthesis and increased the growth rate through cell proliferation and increased the rate of ribosomal protein (RP) synthesis by the selective mobilization of RP mRNAs into polysomes. Furthermore, analysis of the phosphorylation status of the main TOR and S6K kinases from the TOR pathway revealed stimulation by ZmIGF or insulin, whereas rapamycin inhibited its activation. Remarkably, a putative maize insulin-like receptor was recognized by a human insulin receptor antibody, as demonstrated by immunoprecipitation from membrane protein extracts of maize callus. Furthermore, competition experiments between ZmIGF and insulin for the receptor site on maize protoplasts suggested structural recognition of the putative receptor by either effector. These data were confirmed by confocal immunolocalization within the cell membrane of callus cells. Taken together, these data indicate that cell growth and cell proliferation in maize depend on the activation of the TOR-S6K pathway through the interaction of an insulin-like growth factor and its receptor. This evidence suggests that higher plants as well as metazoans have conserved this biochemical pathway to regulate their growth, supporting the conclusion that it is a highly evolved conserved pathway.



Mitogens, hormones, and growth factors are produced by living organisms to coordinate their growth and development.[1,2] In most nonphotosynthetic eukaryotes, ranging from yeast to mammals, the ancient and conserved signal transduction TOR-S6K pathway coordinates growth and cell cycle progression by stimulating the G_1−S phase cell transition,[3,4] as well as metabolic processes related to energy level or apoptosis.[5] This signaling process targets the translational apparatus through the TOR pathway by selectively increasing the extent of ribosomal protein synthesis and inducing ribosomal biogenesis.[6,7] The TOR pathway is known to be activated by environmental signals, nutrients, and stress, as well as by insulin or members of the insulin-like growth factor (IGF) family.[1,8−11] In plants, evidence has shown that the TOR protein has been conserved throughout evolution,[12−15] and it is further involved in growth control and cell proliferation.[16−18] Most of the components of this pathway, such as TOR, RAPTOR, FKBP12, and S6K1 and S6K2 kinases, have also been reported for *Arabidopsis*.[19−21] In maize, orthologs of TOR[13] and S6K proteins[22] have been reported, as well. Other members of this pathway, including the translationally controlled tumor protein (TCP),[23] the Mei2-like protein,[24] the lethal with Sec Thirteen 8/G protein b subunit-like (LST8/GbL)[25] that interacts with Raptor, and the Tap46 subunit in complex with the PP2A kinase,[26] have been reported to regulate growth in the context of the TOR pathway in plants.

IGFs are small signaling peptides widely present in animal cells. They show diversification according to tissue or developmental stage, even though their role in growth control and development is conserved.[27] In plants, several peptide molecules (systemin, clavata3, and phytosulfokines)[28] have been reported to be involved in cell proliferation,[29] but the functional role of these proteins has not been linked to the TOR pathway. In contrast, some reports have demonstrated the existence of peptides recognized by insulin antibodies present in dicocotyledoneous[30,31] and monocotyledoneous[32] plants, suggesting the presence of IGFs in different plants. However, their biochemical and physiological properties have not been fully described or linked to the TOR pathway, but some reports of members of the IGF family, such as the mammalian epidermal growth factor (mEFG), have demonstrated an increased rate of growth of sorgum seedlings.[33] In *Solanum tuberosum* (potato) and *Arabidopsis* plants, the EBP homologue also regulates organ growth by stimulating ribosome biogenesis[34] through the TOR pathway.[35] In maize, a 5.7 kDa peptide, recognized by the human insulin antibody, named *Zea mays* insulin-like growth factor (ZmIGF) has been

Received: March 26, 2013
Revised: December 2, 2013
Published: December 10, 2013

dx.doi.org/10.1021/bi401474x | *Biochemistry* 2013, 52, 9129−9140

purified from embryos, demonstrating that its tertiary structure is similar to that of insulin as indicated by circular dichroism.[36] This peptide has biochemical characteristics that suggest its role in the TOR pathway.[32] Indeed, either insulin or ZmIGF induces maize seedling growth and ribosomal protein synthesis,[16,37] as well as ribosome biogenesis.[38] Furthermore, ZmIGF has been shown to regulate cell growth by cell proliferation in maize tissues,[39,40] suggesting that the biochemical and functional properties are quite similar between plant (ZmIGF) and animal (insulin or IGFs) growth factors. This work provides further evidence of a highly conserved TOR-S6K pathway in maize that regulates growth along with cell division and reveals a putative cell membrane receptor that interacts with either ZmIGF or insulin.

EXPERIMENTAL PROCEDURES

Plant Material and Culture Conditions. Maize (*Z. mays* L.) seeds from Tuxpeño Mexican race var. Costeño were used to establish cultures of maize calli. Excised maize embryos from mature quiescent or immature seeds 15−18 days after pollination were used as the material for induction of slow-growing (SG) and fast-growing (FG) calli, respectively. SG and FG calli were established and maintained using a procedure derived from a combination of the methods described in ref 41. Briefly, mature and immature embryos were placed on induction medium (N6i) that contained N6 basal salts and vitamins, 3% sucrose, 2.3 g/L proline, 200 mg/L hydrolyzed casein, 2 mg/L 2,4-dichlorophenoxy acetic acid, and 3.3 g/L gellan (Sigma-Aldrich, St. Louis, MO) to solidify the medium. The pH was adjusted to 5.7 before the gellan was added. After 3−4 weeks, the callus formed from the scutellum was separated and subcultured on the previously described N6i medium supplemented with 0.1 mg/L kinetin; this medium was called proliferation medium (N6p) and was used for callus maintenance. Cultures were kept at a temperature of 25 ± 2 °C in the dark.

Treatment of Maize Calli with ZmIGF or Insulin. Maize SG calli were grown in solid N6p medium in the dark and incubated with 200 microunits/mL (7 ng/mL) ZmIGF or insulin (SG-Zm or SG-I, respectively). For 5 weeks, the cultures were transferred every week into fresh medium and given a fresh dose of ZmIGF or insulin. Control cultures of SG and FG calli were maintained under the same culture conditions without the addition of ZmIGF or insulin. At the end of the experiments, the calli were flash-frozen in liquid nitrogen and stored at −80 °C until they were used.

Reagents. Bovine insulin and rapamycin were purchased from Sigma-Aldrich, and HeLa cell protein extract was purchased from Millipore.

ZmIGF Purification and Sequencing. ZmIGF was obtained from total soluble protein extract from germinated maize embryonic axes by anionic, cationic, and affinity chromatography using an insulin antibody as a recognition ligand as previously described.[36] The purified peptide demonstrated a single peak by reverse phase high-performance liquid chromatography analysis and a molecular mass of 5700 Da.

ZmIGF was subjected to *de novo* sequencing at the Facultad de Medicina of the Universidad Nacional Autonoma de Mexico by using typical LC−MS/MS workflows employing trypsin as a protease. The peptide was fragmented using both CID and ETD, and an Orbitrap mass spectrometer was set to acquire an MS1 scan at 60000 resolution from m/z 300 to 2000, followed by seven CID or ETD MS2s for the top seven most intense ions. Dynamic exclusion was set for three repeat counts with an ion exclusion list size of 500 and repeat and exclusion durations of 30 s. A 70 min linear gradient from 5 to 40% ACN was used to chromatographically separate peptides prior to their introduction into the mass spectrometer. Spectra were searched against MSDB (http://ftp.ncbi.nih.gov/repository/MSDB) and the B73 maize genome translations (http://ftp.maizesequence.org/current/working-set/) using PEAKS with the decoy fusion method. Two of the resulting peptides aligned with a protein already deposited in GenBank as entry P81009. This ZmIGF sequence was subjected to alignment with the sequences corresponding to the B chain from insulin, IGF-I, IGF-II, and IGF-mini using the ClustalΩ tools site (http://www.ebi.ac.uk); the same amino acid sequences were used to obtain a three-dimensional representation of each peptide. The following Protein Data Bank (PDB) entries were used to represent the structures: 1MSO (insulin), 2GFI (IGF-I), 1IGL (IGF-II), and 1TGR (IGF-mini). PyMOL (http://www.pymol.org) was used to generate the images.

Growth Rate Evaluation. FG and SG maize calli were cultured in liquid N6p medium without (control) or with 200 microunits/mL (7 ng/mL) ZmIGF (SG-Zm) or insulin (SG-I) under the same conditions as indicated above. This material was used to evaluate the growth rate. The fresh weight of each sample was determined for these calli before they were dried at 60 °C to establish the dry weight. The dry weight was measured after calli had been desiccated at 60 °C up to the steady-state weight, and these values were used to calculate the dry:fresh weight ratios. The experiments were performed in triplicate and the results subjected to statistical analysis.

Endogenous Content of ZmIGF. A Western blot assay was used to detect ZmIGF in total soluble protein extracts from FG, SG, SG-Zm, and SG-I calli after they had been cultured for 5 weeks without the addition of exogenous growth factors (ZmIGF or insulin). Equal amounts of total soluble protein from each callus type were resolved via 15% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and transferred onto a PVDF membrane (Millipore). The membrane was incubated overnight at 4 °C with a polyclonal antibody directed against human insulin (Santa Cruz Biotechnology) diluted 1:5000. Subsequently, the PVDF membrane was incubated with a secondary HRP-conjugated antibody (Millipore) diluted 1:5000 and revealed by reagents in the Immobilon Western kit (Millipore) and X-ray film. The densities of the bands were evaluated using ImageJ version 1.36b (http://rsb.info.nih.gov/ij/) and expressed as bar histograms. The experiments were performed in triplicate and the results subjected to statistical analysis.

Semiquantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) for the Evaluation of Cell Cycle Genes. Total RNA from each callus type was used to evaluate the expression of selected genes. For each gene, forward and reverse oligonucleotides were designed and synthesized at the Molecular Unit of the Cellular Physiology Institute of the Universidad Nacional Autonoma de Mexico. Table 1 of the Supporting Information contains the sequences of the designed oligos used for each gene. Analysis by RT-PCR was performed using 100 ng of total RNA with the SuperScript One-step RT-PCR kit (BRL, Life Technologies/Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Amplification conditions were established for each evaluated gene, and the number of cycles was set to linearity to obtain a semi-

quantitative measurement of the mRNA content (Figure 1 of the Supporting Information). Experiments were performed in triplicate and the results subjected to statistical analysis.

DNA Synthesis Evaluation. DNA synthesis was evaluated as previously described.[39] Briefly, maize calli cultured for 5 weeks with 200 microunits/mL (7 ng/mL) ZmIGF or insulin or without the growth factor (control) were incubated for 2 h with 10 μCi/mL of [^{3}H]thymidine [specific activity of 3.18 TBq/mmol (Amersham Biosciences)]. For animal tissue evaluation, 300 mg of adult Wistar rat liver was incubated for 48 h in 2 mL of Krebs-Ringer medium without (control) or with 200 microunits/mL (7 ng/mL) ZmIGF or insulin in the presence of 10 μCi/mL of [^{3}H]thymidine (specific activity of 3.18 TBq/mmol). DNA was extracted from each tissue with the DNA purification kit (PUREGENE DNA, Purification System, Minneapolis, MN) and quantified by spectrometry, and the incorporation of [^{3}H]thymidine was assessed using a liquid scintillation counter (LS 6500, Beckman Coulter).

[^{14}C]Glucose Uptake. Glucose incorporation was measured according to the method described in ref 42. Briefly, 300 mg of adipose tissue obtained from an adult male Wistar rat that had been without food for 18 h was incubated for 1 h at 37 °C under slow agitation, in 2 mL of Krebs-Ringer medium with 25 μCi of [^{14}C]glucose [specific activity of 3.0 TBq/mmol (Amersham)] in the presence of either ZmIGF or insulin (200 microunits each) or without any growth factor as a control; after centrifugation, the amount of [^{14}C]glucose was measured in the supernatant with an LS 6500 scintillation counter from Beckman.

TOR and S6K Phosphorylation Status. To evaluate the phosphorylation status of the TOR and S6K proteins, FG, SG, SG-Zm, and SG-I calli were obtained as described above. In parallel experiments, rapamycin (100 nM) was added to the culture medium and the mixture incubated for an additional 24 h. Total phosphorylated proteins from each callus type were obtained from total soluble protein extracts using the PhosphoProtein purification kit (QIAGEN, GmbH, Hilden, Germany) following the manufacturer's instructions. Phosphorylated proteins were resolved via 10% SDS−PAGE, transferred onto a PDVF membrane (Millipore), and incubated with either phospho (anti-TOR phospho S2448 and anti-p70 S6K Thr 389 from Abcam and Santa Cruz Biotechnology, respectively), or total (anti-mTOR and anti-p70 S6K C-18 from Abcam and Santa Cruz Biotechnology, respectively) antibodies diluted 1:5000 in parallel overnight assays at 4 °C, followed by secondary antibody HRP-conjugated incubation (1:10000 dilution) and detection with reagents in the Immobilon Western kit and X-ray film. The band densities were obtained as described in previous paragraphs. Experiments were performed in triplicate and the results subjected to statistical analysis.

***De Novo* Ribosomal and Cytoplasmic Protein Synthesis.** Ribosomal and cytoplasmic protein synthesis was evaluated in FG, SG, SG-Zm, and SG-I maize calli as described in ref 43. Briefly, maize calli cultured for 5 weeks with 200 microunits/mL (7 ng/mL) ZmIGF or insulin or without a growth factor (control) were incubated for 2 h with 400 μCi of [^{35}S]methionine [specific activity of 43.475 TBq/mmol (Perkin-Elmer, Boston, MA)]. Ribosomal and cytoplasmic proteins were extracted, and [^{35}S]methionine incorporation was measured using an LS 6500 liquid scintillation counter from Beckman.

mRNA Selective Mobilization. To evaluate the ribosomal mRNA selective mobilization, ribosome complexes were isolated according to method described in ref 37. Briefly, total soluble FG, SG, SG-Zm, and SG-I callus protein extracts were centrifuged through a 60% sucrose cushion to obtain ribosome complexes. These complexes were dissolved and fractionated by centrifugation in a sucrose density gradient [15 to 60% (w/v)]. Fractions were separated using a gradient fractionator, and the absorbance of each fraction (at 254 nm) was measured and used to construct ribosomal complex profiles. Fractions comprising monosome (7−9) and polysome (15−17) peaks were pooled, and mRNA was isolated using the Trizol reagent. These RNAs were used to evaluate the mRNA content of the *p0* and *sA* ribosomal proteins by RT-PCR. A kinetic reaction was performed for each product to show that decrease in the linear range (Figure 2 of the Supporting Information).

Cell Membrane Isolation and Plant Receptor Immunoprecipitation. Membranes were obtained from FG maize calli as follows. Thirty grams of tissue was homogenized for 20 s in a blender with extraction buffer [620 mM sorbitol, 50 mM HEPES/BTP (pH 7.8), 15 mM β-mercaptoethanol, 5 mM ascorbic acid, 3 mM EDTA, 1 mM DTT, 1 mM KCl, 0.6% PVP, 0.2% BSA, and complete protease inhibitor cocktail (Roche) in a 1:3 (w/v) proportion]. The homogenate was filtered through four layers of gauze and centrifuged at 11200*g* and 4 °C for 15 min. The supernatant was further centrifuged at 40000*g* and 4 °C for 80 min. The pellet was solubilized in a buffer containing 350 mM sorbitol, 2 mM HEPES/MES (pH 7.6), 1 mM DTT, 1 mM KCl, and 1.6 mg/mL complete protease inhibitor cocktail.[44] Fifty micrograms of maize membrane extract and total HeLa cell protein extract (Millipore) was immunoprecipitated using the Co-IP kit (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions using an antibody against the human insulin receptor (Santa Cruz Biotechnology). Immunoprecipitated proteins were resolved via 10% SDS−PAGE and transferred to a PVDF membrane. The Western blot was performed as described previously using the same antibody against the human insulin receptor diluted 1:3000 and secondary HRP-conjugated antibody diluted 1:10000. Albumin was used as negative control to confirm antibody specificity.

Insulin and ZmIGF Competition Assay. Protoplasts were used to determine ZmIGF and insulin competition for the ZmIGF receptor site. These protoplasts were obtained from FG calli by incubating cell suspension cultures in CPW medium[45] supplemented with 13% manitol and digestion enzymes Meicelase (2%) (Sigma) and macerozyme R10 (0.1%) (Phyto Technology Lab, Shawnee Mission, KS). Cells were left overnight at room temperature while being slowly agitated. Protoplasts were recovered by centrifugation at 80*g* for 10 min; the supernatant was removed, and protoplasts were resuspended in CPW medium supplemented with 21% sucrose. Insulin was bound to biotin using the ImmunoProbe Biotinylation Kit according to the manufacturer's instructions (Sigma-Aldrich). Variable protoplast culture volumes were spin down by brief centrifugation to obtain a cell package of 500 μL for each experimental set. These protoplasts were saturated with 1000 microunits (35 ng) of biotinylated insulin in 1 mL of N6p medium for 30 min (Figure 3 of the Supporting Information). After the incubation, the medium was removed and substituted with fresh N6p medium with increasing ZmIGF doses for 30 min. The amount of biotinylated insulin in the

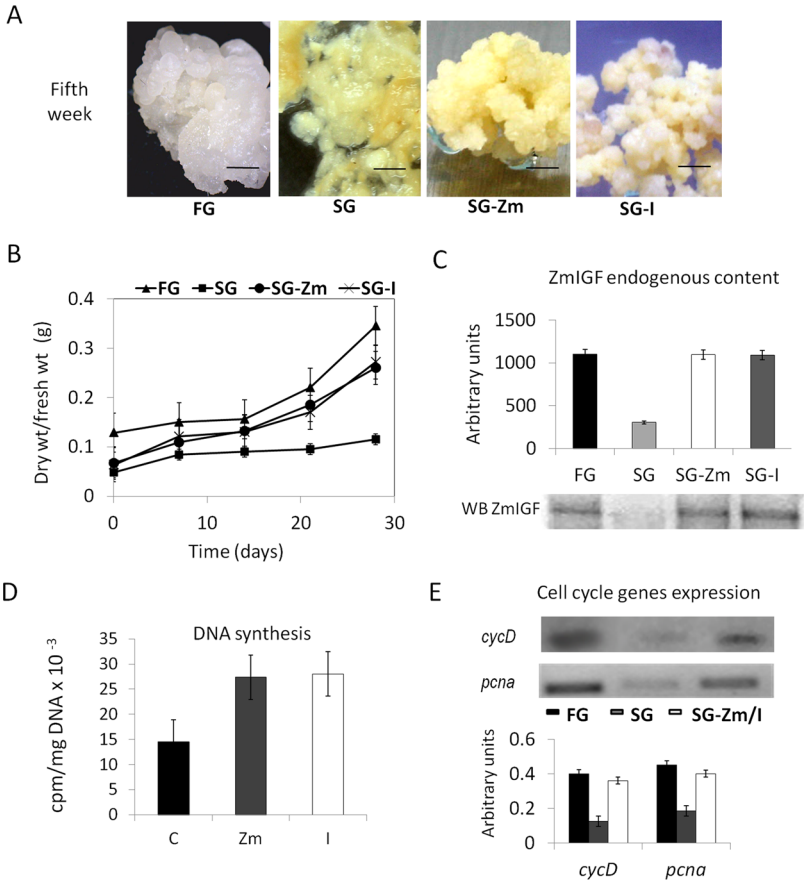


Figure 1. Morphological and growth characteristics of FG, SG, SG-Zm, and SG-I maize calli. The calli were cultivated with or without the addition of ZmIGF or insulin (200 microunits/mL). (A) Morphology of the callus after it had been cultured for 5 weeks. Scale bars are 1 mm. (B) The maize calli growth rate was evaluated every week for 4 weeks. The data represent the average value of the dry:fresh weight ratios of three independent experiments. (C) Endogenous ZmIGF content. FG, SG, SG-Zm, and SG-I calli were further cultivated for 5 weeks under the same conditions without exogenous addition of ZmIGF or insulin. The Western blot, on total soluble protein extracts, was performed using human insulin antibody to detect the ZmIGF signal. (D) DNA synthesis of maize calli during the fifth week of stimulation. Incorporation of thymidine into new DNA was measured by count scintillation. (E) The expression of cell cycle genes *cycD* and *pcna* was evaluated by RT-PCR using total RNA from FG, SG, SG-Zm, and SG-I calli. RT-PCR kinetics (Figure 1 of the Supporting Information) demonstrates that the reaction falls within the linear range of each product. The histogram bars represent band densities as measured by ImageJ version 1.36b.

supernatants for each ZmIGF concentration was determined using the avidin−HABA assay according to the manufacturer's instructions (Sigma-Aldrich).

For the time competition experiments, protoplasts were incubated with a fixed dose (400 microunits/mL) of biotinylated insulin to saturate the membrane receptors and incubated at room temperature for 30 min while being slowly agitated. The protoplasts were then spun down to remove the medium, and an equal volume of fresh medium containing 400 microunits/mL ZmIGF was added to the protoplasts saturated with the opposite factor. The biotinylated insulin content was evaluated in the supernatant of each protoplast set every 5 min for up to 30 min. Control experiments included protoplasts that were saturated with biotinylated insulin and further incubated with the corresponding volume of N6P medium without the competitor (ZmIGF).

ZmIGF Receptor Immunolocalization. To identify the location of the ZmIGF receptor, maize callus cells were washed twice with PBS, blocked with 2% BSA in PBS (blocking buffer) for 1 h, and incubated for 2 h with an antibody directed against the human insulin receptor (Santa Cruz Biotechnology) diluted 1:40 in blocking buffer, washed once with PBS, and subsequently incubated with the secondary Alexa Fluor 488-conjugated antibody diluted 1:25 in blocking buffer (Sigma) for 1 h, washed twice with PBS, and fixed with acetone for 30 min at −20 °C. Cells were washed once more with PBS and mounted with a drop of glycerin as the mounting medium on a glass slide with a coverslip that was sealed with nail polish. HeLa cells were grown onto coverslips for 24 h, washed once with PBS, fixed with 3% formaldehyde in PBS for 30 min, washed three times with PBS, blocked with 10% fetal bovine serum in PBS (blocking buffer) for 60 min, incubated with human insulin receptor antibody diluted 1:50 in blocking buffer, washed once with PBS, incubated with secondary antibody diluted 1:50 for 30 min, and mounted as described for maize cells. Fluorochromes propidium iodide [1 μg/mL (Figure 6)] and calcofluor [1 μg/mL (Figure 4 of the Supporting Information)] (Sigma-Aldrich) were applied to the mounting medium to visualize the cell nuclei, cell membrane, and cell wall. Negative controls were obtained by incubating the maize cells only in presence of the secondary antibody; no signal was observed, and this indicated the absence of the specific interaction of this antibody (Figure 5 of the Supporting Information). Cells were viewed using an Olympus FLUOVIEW FV1000 laser-scanning confocal microscope to detect the fluorescent signal at the Unit of Microscopy

dx.doi.org/10.1021/bi401474x | *Biochemistry* 2013, 52, 9129−9140

of Facultad de Química of the Universidad Nacional Autonoma de Mexico. The green fluorescent signal of the Alexa Fluor 488 conjugate was observed at the cell membrane. Alexa Fluor 488 fluorescence was excited with a diode laser at 473 nm, and emission was detected between wavelengths of 485 and 585 nm. Propidium iodide was excited at 559 nm, and emission was detected between 570 and 670 nm. Calcofluor was excited at 405 nm, and emission was detected between wavelengths of 405 and 520 nm. FV-10-ASW version 1.7 was used to generate and process the images.

Statistical Analysis. The Student's *t* test was used to obtain standard deviations in the required experimental calculation.

RESULTS

Functional Similarities between ZmIGF and Insulin. Previous findings have shown that maize seedling embryos express a small 5.7 kDa peptide, known as ZmIGF.[32,36] This growth factor was found to share some biological functional similarities with insulin, because both effectors stimulate germination and seedling growth in maize[32] through the TOR pathway.[16] Thus, to further examine the effects of ZmIGF and insulin on maize tissue growth, cultures of FG or SG calli were used as a model system. The SG callus was stimulated with either effector (ZmIGF or insulin), and the FG callus was used as a positive control. At the beginning of the stimulation, SG calli appeared as a yellow to brown colored cell mass with a watery and loose consistency and FG calli appeared as a white compact cell mass. After being cultured for 5 weeks without the addition of any growth factor, both control calli, FG and SG, maintained their original morphologies. However, SG cultures grown with either ZmIGF or insulin (SG-Zm or SG-I, respectively) showed a dramatic morphological change that resembled FG calli (Figure 1A). The growth rate was evaluated by determining dry:fresh weight ratios. The SG-Zm:SG-I growth ratio increased four times with respect to that of the control SG calli, reaching values similar to those found for the FG calli after they had been cultured for 5 weeks, with either insulin or ZmIGF (Figure 1B). Further, DNA synthesis was also evaluated on these calli, revealing an increase of 2 orders of magnitude in this parameter in SG calli stimulated with either ZmIGF or insulin (Figure 1C). Moreover, the expression of cell cycle genes, *cycD* and *pcna*, was evaluated by RT-PCR in the linear range of each product, using total RNA from SG, FG, and SG-Zm/SG-I calli. Results showed that the level of expression of these genes significantly increased in the stimulated calli (Figure 1D), supporting the conclusion that growth was due to cell proliferation, rather than to cell enlargement. Previous results from our research group have already proven that cell division in calli tissues occurs by stimulation with ZmIGF or insulin,[39] and recent evidence also showed that this also happens in maize seedlings, through the promotion of the G1−S1 phase transition during the cell cycle.[40] These data indicate a phenomenon similar to that documented for animal cells in response to insulin.[3,46] To determine the endogenous growth factor content of these calli, Western blot analysis were performed using an antibody raised against human insulin that was previously demonstrated to recognize the purified ZmIGF.[36] Results revealed that the FG calli contained a high level of ZmIGF, whereas the SG calli contained a low concentration of this growth factor. Nevertheless, after being cultured for 5 weeks, the SG-Zm- and SG-I-stimulated calli demonstrated an endogenous ZmIGF content similar to that of FG calli (Figure 1E). These values were maintained even after the calli had been cultured for several weeks without further addition of exogenous growth factor, suggesting that the growth of FG, SG, SG-Zm, and SG-I calli is related to their endogenous growth factor content.

The data described above suggest that the functional similarities between ZmIGF and insulin on plant tissues are most probably due to common tertiary structural bases of the two effectors. To further inquire about this phenomenon, the effect of ZmIGF was tested on insulin-sensitive animal tissues. To this end, the rate of incorporation of [^{14}C]glucose into the rat adipose tissues, stimulated by either ZmIGF or insulin, was performed. Results indicated that ZmIGF was capable of inducing the uptake of glucose into the adipose rat cells to the same extent as insulin did (Figure 2A). In a similar experiment,

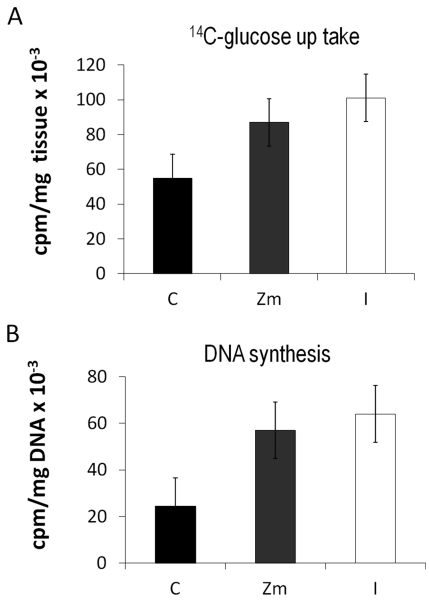


Figure 2. Functional equivalence of animal and plant growth factors. (A) Uptake of [^{14}C]glucose from adipose rat cells by activation of either ZmIGF or insulin. (B) Stimulation of DNA synthesis on rat liver tissue caused by the effect of the growth factors evaluated by the incorporation of [^{3}H]thymidine into synthesized DNA.

DNA synthesis was also assessed by [^{3}H]thymidine incorporation in rat liver tissues after stimulation by either ZmIGF or insulin (Figure 2B). Results again corroborated the functional similarities between ZmIGF and insulin, strongly suggesting structural similarities, as well.

ZmIGF Adopts an Insulin-like Folding Structure. The ZmIGF peptide was purified by an already established procedure[36] (Figure 3A) and subjected to sequencing by mass spectrometry. Considering that the insulin-like superfamily (insulin, IGF-I, IGF-II, relaxin, and bombyxin) folding behavior is controlled by their B chain domain,[47] the obtained ZmIGF amino acid sequence was aligned with the B chain from insulin as well as with the equivalent B chain of IGF-I, IGF-II, and IIGF-I-mini (a synthetic IGF formed by domains A and B of IGF-I[47]). The alignment showed few similarities among ZmIGF, insulin, and the others IGFs, mainly at the cysteine residues (Figure 3B). Circular dichroism analysis in the far-UV region of insulin,[48] IGF-I,[47] and ZmIGF[36] has already shown great tertiary structure similarities among these molecules, particularly on their predominant α-helix structure. On the basis of this information, a model of the correspondent B chain

dx.doi.org/10.1021/bi401474x | *Biochemistry* 2013, 52, 9129−9140

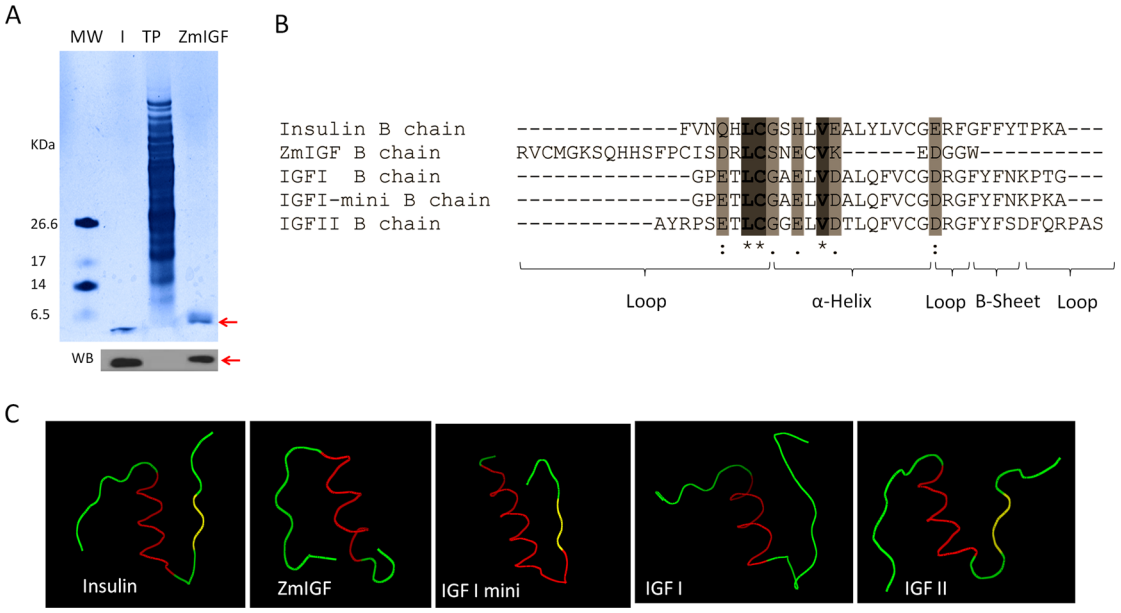


Figure 3. ZmIGF plant growth factor is structurally similar with members of the IGF family. (A) ZmIGF detection in E callus total protein extract after purification, where insulin (I) was used as a positive control. Abbreviations: MW, molecular weight marker; TP, total protein; WB, Western blot detection. Arrows denote the ZmIGF peptide. (B) Amino acid sequence alignment of chain B of insulin and IGFs with ZmIGF. Bold letters and dark shading denote identical residues; light gray shading denotes equivalent amino acid substitutions. (C) Three-dimensional representation of amino acids used for alignment. Structural information was obtained from PDB coordinates of PDB entries 1MSO (insulin), 2GFI (IGF-I), 1IGL (IGF-II), and 1TGR (IGF-mini). PyMOL (http://www.pymol.org) was used to obtain the images. In the structures, loops are colored green, α-helices red, and β-sheets yellow.

of the ZmIGF peptide was obtained using the SWISS MODEL Web site (http://beta.swissmodel.expasy.org/). The model provided a three-dimensional representation of these molecules that showed the structural similarity between the B chains from ZmIGF and those from insulin as well as the corresponding chains from the other IGFs (Figure 3C), particularly on the α-helix structure that was predominant (Figure 3C).

Ribosomal Protein Synthesis and Selective Mobilization of mRNA into Polysomes. TOR pathway activation is known to selectively mobilize ribosomal protein mRNAs into polysomes for translation.[49] This process occurs to support the ribosome biogenesis that is required for organisms to grow. Thus, to corroborate that both factors, ZmIGF and insulin, stimulate the same pathway, we performed the following experiments. The effect of ZmIGF and insulin on *de novo* ribosomal protein synthesis was evaluated using [^{35}S]-methionine in the maize callus experimental model. Results indicate that *de novo* ribosomal protein synthesis between control FG and SG calli showed higher and lower extents of [^{35}S]methionine incorporation, respectively (Figure 4A). This result correlated with the fast and slow growth rate of each callus tested. After stimulation with ZmIGF or insulin, the SG-Zm and SG-I calli demonstrated in both cases a large increase in the extent of *de novo* ribosomal protein synthesis (Figure 4A), whereas cytoplasmic proteins were not significantly altered in any type of callus (Figure 4B). To further analyze this phenomenon, ribosomal complexes from FG, SG, SG-Zm, and SG-I maize calli were extracted and fractionated by ultracentrifugation in a 15 to 60% sucrose density gradient (Figure 4C,D). Ribosome profiles demonstrated a high polysome content for the FG, SG-Zm, and SG-I calli and a low content for the SG calli, indicating that an increase in the polysomal fraction was associated with a faster growth rate because of the effect of the stimulating agent. Furthermore, mobilization of ribosomal protein mRNA into polysomes was evaluated. Thus, mRNAs from the monosomal and polysomal fractions of each type of callus were purified and used to evaluate the expression of two genes encoding ribosomal proteins by RT-PCR in the linear range of each product (Figure 2 of the Supporting Information). We found that levels of mRNA from ribosomal protein genes *p0* and *sA* in the polysomal fraction were high in the FG calli and low in the SG calli. After stimulation with the effectors mentioned above, these mRNAs also demonstrated high values in the polysomal factions from SG-Zm and SG-I (Figure 4E). To further corroborate these findings, the gene expression levels of ribosomal protein genes *p0* and *sA* were evaluated within the total RNA from each type of callus. Results demonstrated no change in the total amount of these mRNAs (Figure 4F), indicating that this phenomenon corresponded to mobilization of specific mRNA into polysomes rather than to increase the level of *de novo* transcription of these mRNAs. Overall, these results support the interpretation that a functional TOR-S6K pathway is present in maize cells and that it is activated by ZmIGF. Thus, in maize, the TOR pathway selectively regulates ribosomal protein synthesis by specific mobilization of their mRNAs into polysomes, which is similar to what has been shown to occur by insulin in several animal models.[2]

TOR-S6K Pathway Activation. Phosphorylation of the central TOR and S6K kinases has been demonstrated to occur after insulin or IGF TOR pathway activation.[1,16,50] To further confirm that the TOR pathway is similarly activated by ZmIGF in maize calli, the phosphorylation status of TOR (Figure 5A) and S6 (Figure 5B) kinases was analyzed by specific antibody recognition in the control (FG and SG) and stimulated (SG-Zm and SG-I) calli. The results demonstrated that either ZmIGF or insulin induced phosphorylation of these kinases. After rapamycin had been added to the system, strong TOR

dx.doi.org/10.1021/bi401474x | *Biochemistry* 2013, 52, 9129−9140

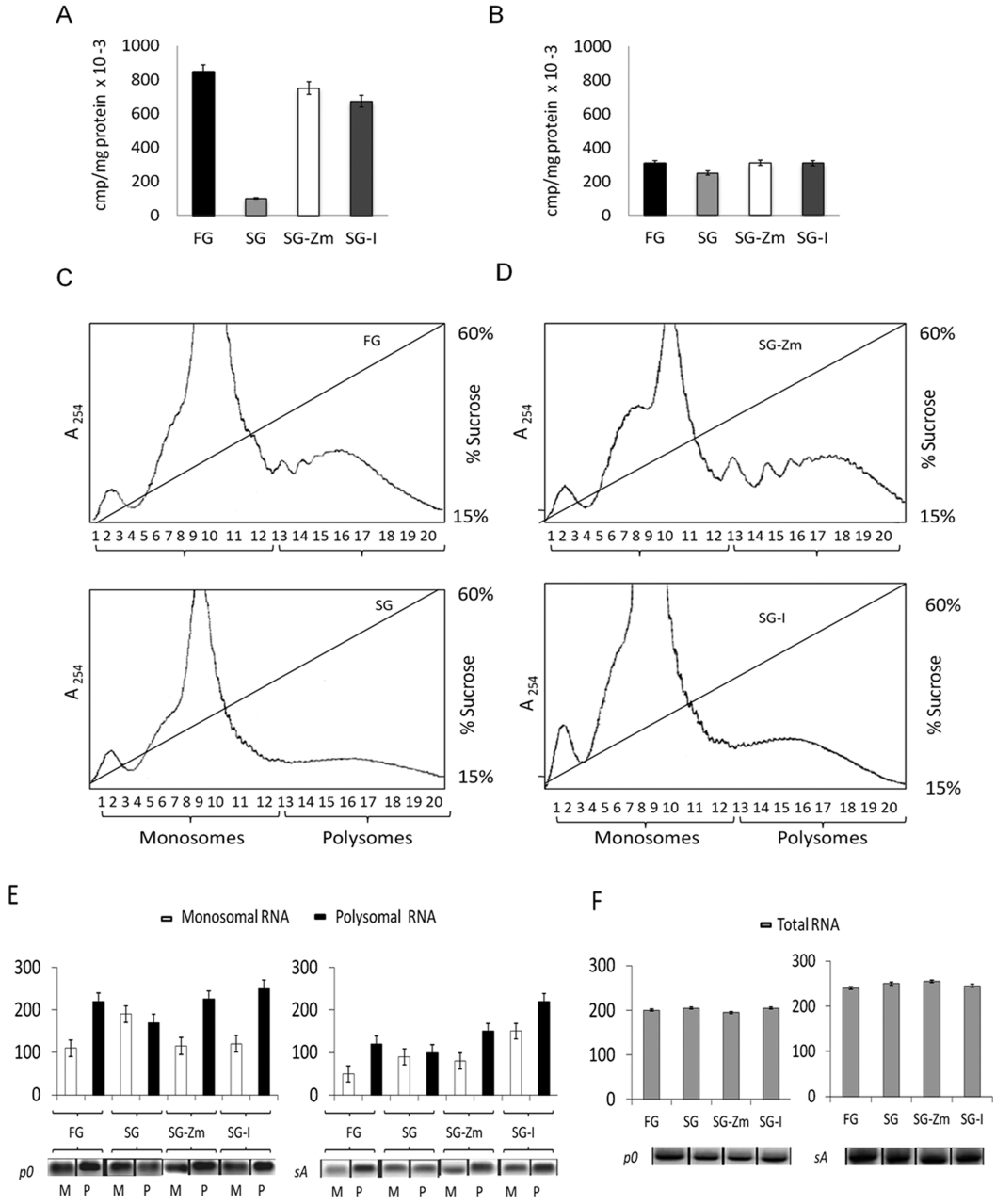


Figure 4. Translational activation induced by ZmIGF or insulin in FG, SG, SG-Zm, and SG-I maize calli. *De novo* ribosomal and cytoplasmic protein synthesis was evaluated by the incorporation of [^{35}S]methionine into new proteins as described in Experimental Procedures: (A) ribosomal and (B) cytoplasmic proteins. Ribosome complexes were fractionated through a density sucrose gradient ranging from 15 to 60%. (C and D) Ribosome profiles of (C) control FG and SG calli and (D) stimulated SG-Zm and SG-I calli. ZmIGF plant growth factor induces selective mobilization of mRNA into polysomes. (E) Expression of ribosomal genes *p0* and *sA* was evaluated by RT-PCR using RNA extracted from monosome (M) or polysome (P) fractions. Fractions 7−9 and 15−17 from sucrose density gradients were pooled to extract mRNA associated with monosomal and polysomal fractions, respectively. (F) Total RNA (T) from each callus type was also used to evaluate ribosomal gene expression. RT-PCR kinetics were performed for each gene (Figure 2 of the Supporting Information), and data represent the linear range of each product. The histograms represent band densities measured with ImageJ version 1.36b. The data represent averages of three independent experiments.

and S6K kinase phosphorylation inhibition was observed (Figure 5A,B), consistent with reports indicating that rapamycin inhibits the TOR pathway in animals[51] and plant systems.[18,22,39] Overall, these data support the statement that ZmIGF is responsible for inducing maize growth through the activation of the TOR pathway by phosphorylating TOR and S6K kinases.

ZmIGF and Insulin Interact with a Putative Plant Insulin-like Receptor. These results confirm the presence of an active TOR pathway in maize tissues that regulates growth. Further, it implies the presence of a plant insulin-like receptor that transduces the external signal when interacting with the ZmIGF effector. To test this postulate, FG callus cell membrane protein extracts (C) as well as HeLa cell total protein extracts (H), used as a control, were obtained and immunoprecipitated with a commercial antibody raised against the human insulin receptor (IR). It is worth mentioning that the commercial IR antibody (Santa Cruz Biotechnology) used in these experiments was designed against a peptide at the N-terminus of the human insulin receptor that recognizes the

dx.doi.org/10.1021/bi401474x | *Biochemistry* 2013, 52, 9129−9140

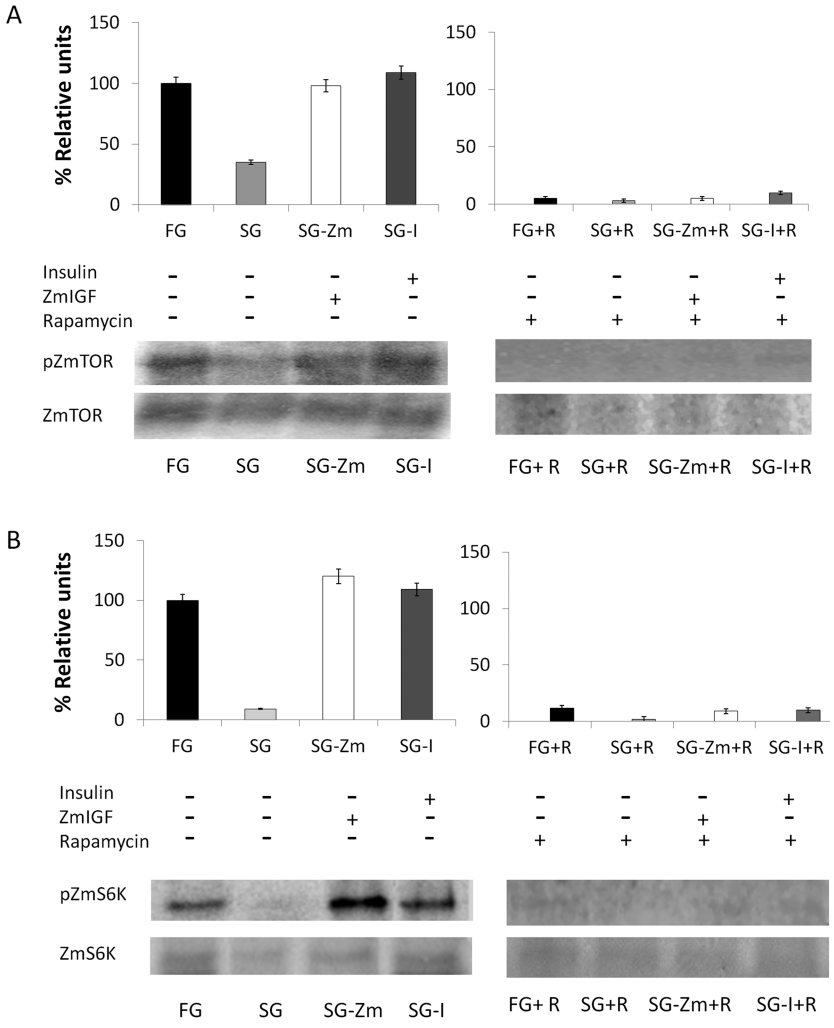


Figure 5. (A) TOR and (B) S6K phosphorylation on FG, SG, SG-Zm, and SG-I maize calli. Total soluble and phosphorylated proteins were analyzed by a Western blot using specific antibodies for the phosphorylated (pTOR and pS6K) and nonphosphorylated TOR and S6K. Addition of rapamycin to cultures of calli inhibited TOR and S6K phosphorylation. Histograms represent the band densities measured with ImageJ version 1.36b.

external α subunit region but not the cytoplasmic portion of the receptor that contains the kinase domain. The Western blot of the immunoprecipitates showed only a single protein of approximately 55 kDa corresponding to the putative ZmIGF receptor of maize cell membranes. The positive control showed the human IR receptor (125 kDa protein) in the HeLa cell extract, corroborating the antibody specificity (Figure 6A). To further confirm the specific interaction between ZmIGF or insulin and a putative plant insulin-like receptor, we performed competition assays. Toward this end, maize protoplasts were obtained from FG maize calli and incubated with biotinylated insulin up to saturation of the putative membrane receptor sites (Figure 3 of the Supporting Information). These protoplasts were then centrifuged and used for two different sets of experiments. In the first set, the protoplasts already saturated with biotinylated insulin were incubated at room temperature for 30 min in a fixed volume with increasing concentrations of ZmIGF to displace the previously attached factor. After a fixed incubation period, the protoplasts were centrifuged and the amount of biotinylated insulin released into the supernatant was determined for each experimental set (Figure 6B). Results indicated that ZmIGF is able to displace the biotinylated insulin attached to the callus cell membranes, as a quantitative function relative to the control lacking the competitor (ZmIGF). In the second experiment, maize protoplasts already saturated with biotinylated insulin were incubated with a fixed dose (400 microunits/mL) of ZmIGF or none. The release of biotinylated insulin into the medium was then evaluated at different time periods (Figure 6C). Results indicate that the biotinylated insulin already attached at the putative membrane receptor site was displaced by the maize growth factor faster than the control, in the absence of the growth factor that shows the passive release of biotinylated insulin into the medium.

Membrane Receptor Immunolocalization. On the basis of the results presented above, immunolocalization experiments were performed to inquire about the presence of such a receptor on intact cell membranes. To this end, FG maize cell suspensions were obtained and incubated with the IR antibody used above and a fluorescent secondary antibody. The plant ZmIGF putative receptor signal was localized by confocal microscopy at the maize cell membrane as the green fluorescent dots present at the periphery of the cells (Figure 6Da).

Notice that this signal coincided with the red fluorescence signal from propidium iodide that identifies the maize cell membrane (panels b and c of Figure 6D). In addition, another set of FG callus cells was incubated under the same conditions

dx.doi.org/10.1021/bi401474x | *Biochemistry* 2013, 52, 9129−9140

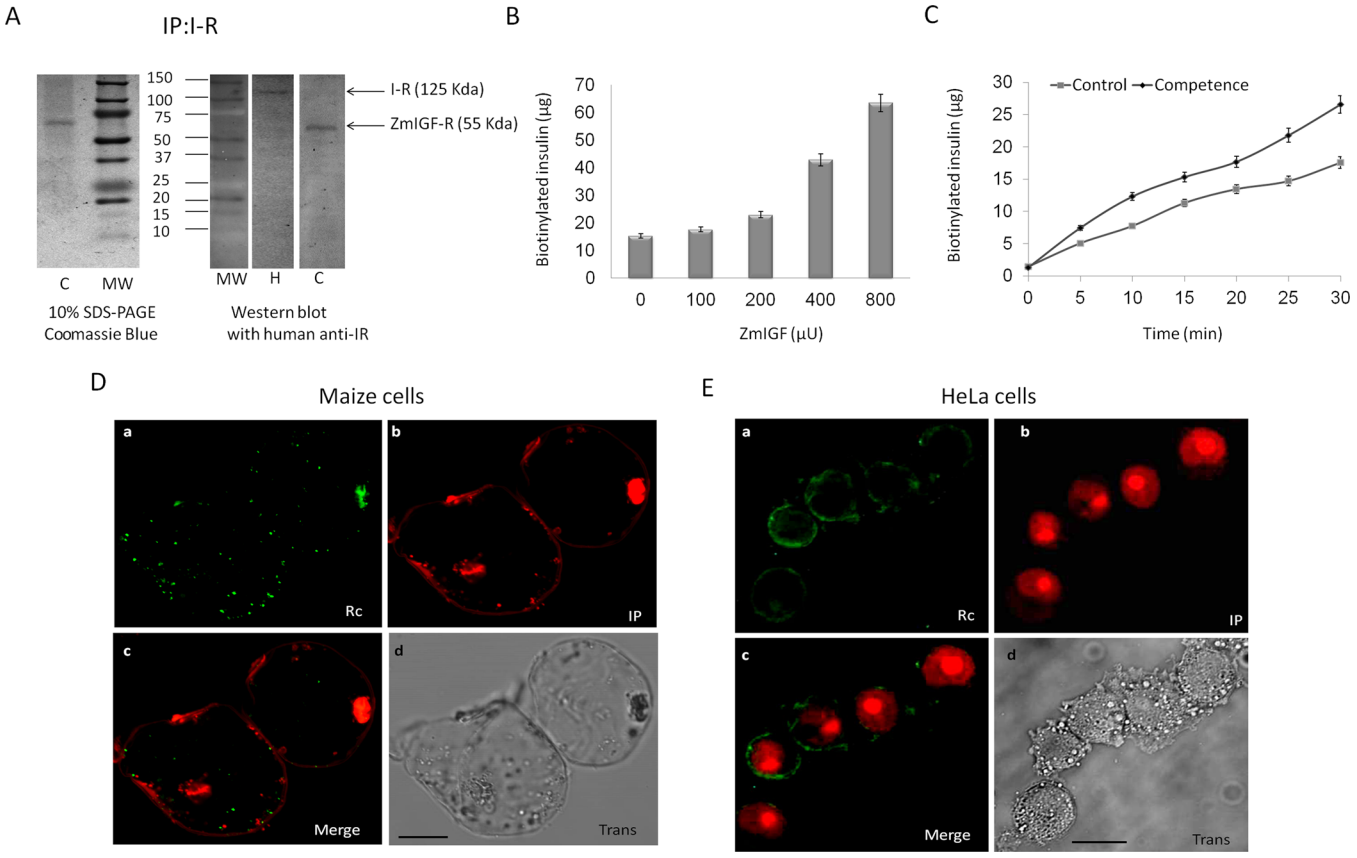


Figure 6. ZmIGF and insulin interact with a putative maize insulin-like membrane receptor. (A) Immunoprecipitation was performed with maize cell membrane extract and insulin with antibody against human insulin receptor. The Western blot showed recognition of a putative receptor in membrane protein extracts from maize callus tissues (C). HeLa cell protein extracts (H) were used as a positive control. These results are representative of three independent experiments. Competition assays between ZmIGF and biotinylated insulin for the putative maize insulin-like membrane receptor in maize cells. Maize protoplasts saturated with biotinylated insulin were used for two different experiments. Panel B shows competition by concentration, in which saturated protoplasts were incubated with increasing ZmIGF concentrations. Release of biotinylated insulin into the medium was evaluated. Panel C shows competition by time period, in which saturated protoplasts with biotinylated insulin were incubated with a fixed ZmIGF concentration (400 microunits/mL), and the biotinylated insulin content was evaluated in the supernatant every 5 min for up to 30 min. In the graphic, competence represents the release of biotinylated insulin from cells into the culture medium by the displacement of ZmIGF from the receptor site. Control experiments included protoplasts that were incubated with an equal volume of culture medium without the competitor. Maize membrane receptor immunolocalization was performed by confocal microscopy. Images show cells incubated with an antibody directed against the human insulin receptor and the Alexa Flour 488 secondary antibody. (D) Maize cells with the insulin-like receptor. (a) The green fluorescent signal indicates the location of the maize ZmIGF receptor (Rc) on the cell membranes of calli. (b) Cells stained with propidium iodide (IP) demonstrate red fluorescence at the cell membrane and in the nuclei. (c) The merged signals from panels a and b indicate an overlap of the maize ZmIGF receptor and fluorochrome signals. (d) Cell shape and integrity determined via phase-contrast microscopy (Trans). (E) Control HeLa cells with the insulin receptor. Panels a–d show the same content that is shown for maize cells. Scale bars in Panel D are 20 μm and in Panel E 10 μm. The data represent averages of three independent experiments.

but with the calcofluor flourochrome to detect the cell wall, and a blue fluorescent color was observed at the cell periphery (Figure 4 of the Supporting Information). Phase-contrast images confirmed the structural integrity of the cells (panel d of Figure 6D), which is similar to that of the HeLa cells used as control that showed the receptor is located at the periphery of the cells within the cell membranes (panels a–d of Figure 6E), as reported by the manufacturer of the antibody (Santa Cruz Biotechnology).

Overall, these data support the proposal that a putative ZmIGF receptor is present within the maize cell membrane that is capable of interacting with either ZmIGF or insulin, internalizing this signal to induce cell growth through the activation of the TOR pathway.

■ DISCUSSION

The TOR signaling pathway, a central regulator for cell growth and proliferation in most nonphotosynthetic eukaryotes,[2,4,5,50,52] has also been recognized in plants,[12,13,15,22,35] even though all of the signaling components have not yet been identified in these organisms. In particular, the upstream components of this route, such as the effector and the receptor responsible for activating this pathway, have remained elusive. In this study, a biochemical approach was designed to further dissect the TOR pathway in maize by using the plant insulin-like growth factor, ZmIGF,[36] or insulin as a control, on slow- and fast-growing cell cultures as biological models. Our results confirmed that TOR pathway activation in maize calli is induced by ZmIGF, as revealed by the phosphorylation of TOR and S6 kinases (Figure 5), a finding similar to what has been reported to occur in nonphotosynthetic eukaryotes[1,4,5,50,53] and

dx.doi.org/10.1021/bi401474x | Biochemistry 2013, 52, 9129–9140

plant models.[16,18,35] TOR pathway activation also involves ribosome biogenesis, a well-known process required to sustain growth in animals and plants.[11,35,52] Our results further confirm an increase in the rate of growth through cell division (Figure 1) along with an increase in the rate of ribosomal protein synthesis (RP) by selective mobilization of RP-mRNAs into polysomes induced by either ZmIGF or insulin (Figure 4). This phenomenon has also been demonstrated on insulin-stimulated maize seedlings,[37] as well as in ribosome biogenesis, observed in the same system.[38] Furthermore, insulin and ZmIGF have previously been shown to increase the rate of maize seedling and callus mitosis,[16,39] processes that were recently confirmed to occur by the activation of S phase genes promoting cell cycle progression in maize[40] and *Arabidopsis*[15] tissues.

The identity of IGF-like growth factors in plants is scarce; even though few reports indicate their existence on leguminose,[30,31] maize,[16,36] potato,[34] and *Arabidopsis*,[35] although most of them have not been shown to regulate plant growth through the TOR pathway. Therefore, our results with respect to the functional equivalence between the animal and plant growth factor are relevant. Furthermore, because ligand receptor interaction is the first step in TOR pathway activation, and the ligand structure is particularly relevant for receptor recognition, the insulin structure provided important information. In particular, the B chain α-helix, the main component in the binding surface,[54] and its C-terminal domain influence the conformation of monomeric active insulin that presents biological activity by interacting with its receptor.[55] Therefore, the B chain tertiary structure similarities among the ZmIGF model, insulin, and other members of the IGF family suggest that ZmIGF adopts an insulin-like three-dimensional structure that allows it to be recognized by the receptor in animal cells (Figure 3), stimulating glucose uptake as well as DNA synthesis in adipose tissue (Figure 2). These data strongly suggest that the functional equivalence between ZmIGF and insulin resides in a similar structural conformation rather than a similar sequence, thus allowing the interaction with either receptor.

Circular dichroism data previously reported for ZmIGF[36] and insulin[47] also support this proposal. Furthermore, different folding of insulin into one monomer and IGF1 into two isomers is essential to achieve biological activity; regardless of their sequences,[47,55,56] these findings support the idea that tertiary structure and conformation are more relevant than the amino acid sequence for their function.

In animals, it is well documented that the IR membrane receptor is a homodimer with two identical half-receptor dimers composed of α and β subunits.[57] The α extracellular subunits interact with insulin[55] or IGFs with weak affinity,[47] internalizing the signal for controlling a series of metabolic processes through the TOR-S6K phosphorylation cascade.[2,4,50,52]

In this report, results of immunoprecipitation and immunolocalization by confocal microscopy (Figure 6D,E) as well as competition experiments with ZmIGF and insulin (Figure 6B) have revealed the presence of an IGF-like growth factor (ZmIGF) and a putative IGF-like receptor (Rc-ZmIGF) in maize cells, which is capable of receiving, transducing, and internalizing the growth factor signals (Figures 2, 4, and 5), a finding similar to what has been reported for insulin, IGF-I, IGF-II, and EGF receptors in animal cells.[50,58,59] Moreover, EGF-like binding domains have been found in a cell wall-associated receptor-like kinase (Waki) in *Arabidopsis*,[60] a fact that suggests the existence of an EGF-like ligand; therefore, the putative receptor reported in this research, which is also associated with the cell wall, is quite relevant (Figure 4 of the Supporting Information). Taken together, all these data support the conclusion that the TOR pathway regulates cell growth and proliferation in maize tissues through an effector–receptor system that activates the signaling pathway. Finally, this study strengthens the evidence that higher plants as well as nonphotosynthetic eukaryotes have conserved equivalent biochemical pathways for regulating cell growth and proliferation through similar effector–receptor systems, indicating functional evolutionary conservation of this pathway in eukaryotes from different kingdoms.[5,14,17,18,36,37,61]

■ ASSOCIATED CONTENT

Supporting Information

Forward and reverse oligonucleotide sequences of genes evaluated in this work (Table 1); RT-PCR reaction kinetics of genes *cycD*, *pcna*, *p0*, and *SA*; saturation of protoplasts with biotynilated insulin; and confocal microscopy of immunolocalization of a putative plant IGF receptor negative control and cell wall detection. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Laboratorio 103, Departamento de Bioquímica, Facultad de Química Conjunto "E" Ciudad Universitaria, 04510 México, D.F., México. E-mail: estelas@unam.mx. Phone: (52 55) 56225278. Fax: (52 55) 56225329.

Funding

This study was supported by Grants DGAPA IN 212910 and CONACYT 101327. V.G.-V. was a postdoctoral fellow supported by the DGAPA-UNAM program and the CONACYT project.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Drs. Alberto Huberman and Tzvetanka D. Dimitrova for reading the manuscript and commenting on the revision, Dr. Leon Castilla for commenting on the ZmIGF model, and M. en C. Ma. Teresa Olvera for establishing the *in vitro* cultures.

■ ABBREVIATIONS

TOR, target of rapamycin; ZmIGF, *Z. mays* insulin-like growth factor; SG, slow-growing callus; FG, fast-growing callus.

■ REFERENCES

(1) Abe, Y., Yoon, S. O., Kubota, K., Mendoza, M. C., Gygi, S. P., and Blenis, J. (2009) p90 ribosomal S6 kinase and p70 ribosomal S6 kinase link phosphorylation of the eukaryotic chaperonin containing TCP-1 to growth factor, insulin, and nutrient signaling. *J. Biol. Chem. 284*, 14939–14948.

(2) Ma, X. M., and Blenis, J. (2009) Molecular mechanisms of mTOR mediated translational control. *Nat. Rev. Mol. Cell Biol. 10*, 307–318.

(3) Kozma, S. C., and Thomas, G. (2002) Regulation of cell size in growth, development and human disease PI3K, PKB and S6K. *BioEssays 24*, 65–71.

(4) Ekim, B., Magnuson, B., Acosta-Jaquez, H. A., Keller, J. A., Feener, E. P., and Fingar, D. C. (2011) mTOR Kinase Domain

Phosphorylation Promotes mTOR signaling, Cell Growth, and Cell Cycle Progression. *Mol. Cell. Biol. 31*, 2787−2801.
(5) Liao, X.-H., Majithia, A., Huang, X., and Kimmel, A. R. (2008) Growth control via TOR kinase signaling, an intracellular sensor of aminoacid and energy availability, with crosstalk potential to proline metabolism. *Amino Acids 35*, 761−770.
(6) Shahbazian, D., Roux, P. P., Mieulet, V., Cohen, M. S., Raught, B., Taunton, J., Hershey, J. W., Blenis, J., Pende, M., and Sonenberg, N. (2006) The mTOR/P13K and MAPK pathway converge on eIF4B to control its phosphorylation and activity. *EMBO J. 25*, 2781−2791.
(7) Wang, X., and Proud, C. G. (2006) The mTOR pathway in the control of protein synthesis. *Physiology 21*, 362−369.
(8) Giannakou, M. E., and Partridge, L. (2007) Role of insulin-like signaling in *Drosophila* lifespan. *Trends Biochem. Sci. 32*, 180−188.
(9) Ma, X. M., Yoon, S. O., Richardson, C. J., Jülich, K., and Blenis, J. (2008) SKAR links pre-mRNA splicing to mTOR/S6KI-mediated enhanced translation efficiency of spliced mRNAs. *Cell 133*, 303−313.
(10) Petersen, J. (2009) TOR signaling regulates mitotic commitment through stress-activated MAPK and Polo Kinase in response to nutrient stress. *Biochem. Soc. Trans. 37*, 273−277.
(11) Markou, T., Marshall, A., Cullingford, T., Tham, E., and Clerk, A. (2010) Regulation of the cardiomyocyte transcriptome vs translatome by endothelin-1 and insulin: Translational regulation of 5′ terminal oligopyrimidine tract (TOP) mRNAs by insulin. *BMC Genomics 11*, 1−16.
(12) Robaglia, C., Menand, B., Lei, Y., Sormani, R., Nicolai, M., Gery, C., Teoule, E., Deprost, D., and Meyer, C. (2004) Plant growth: The translational connection. *Biochem. Soc. Trans. 32*, 581−584.
(13) Agredano-Moreno, L. T., Reyes de la Cruz, H., Martínez-Castilla, L. P., and Sánchez de Jiménez, E. (2007) Distinctive expression and functional regulation of the maize (*Zea mays* L.) TOR kinase ortholog. *Mol. BioSyst. 3*, 1−10.
(14) John, F., Roffler, S., Wicker, T., and Ringli, C. (2011) Plant TOR signalling components. *Plant Signaling Behav. 6*, 1700−1705.
(15) Xiong, Y., McCormack, M., Li, L., Hall, Q., Xiang, C., and Sheen, J. (2013) Glucose-TOR signalling reprograms the transcriptome and activates meristems. *Nature 496*, 181−186.
(16) Dinkova, T. D., Reyes de la Cruz, H., García-Flores, C., Aguilar, R., Jiménez-García, L. F., and Sánchez de Jiménez, E. (2007) Dissecting the TOR-S6K signal transduction pathway in maize seedlings: Relevance on cell growth regulation. *Physiol. Plant. 130*, 1−10.
(17) Dobrenel, T., Marchive, C., Sormani, R., Moreau, M., Mozzo, M., Montané, M. H., Menand, B., Robaglia, C., and Meyer, C. (2011) Regulation of plant growth and metabolism by the TOR kinase. *Biochem. Soc. Trans. 39*, 477−481.
(18) Xiong, Y., and Sheen, J. (2012) Rapamycin and Glucose-Target of Rapamycin (TOR) Protein Signaling in Plants. *J. Biol. Chem. 287*, 2836−2842.
(19) Mahfouz, M. M., Kim, S., Delauney, A. J., and Verma, D. P. S. (2006) *Arabidopsis* TARGET OF RAPAMYCIN interacts with RAPTOR which regulates the activity of S6 kinase in response to osmotic stress signals. *Plant Cell 18*, 477−490.
(20) Sormani, R., Yao, L., Menad, B., Ennar, N., Lecampion, C., Meyer, C., and Robaglia, C. (2007) *Saccharomyces cerevisiae* FKBP12 binds *Arabidopsis thaliana* TOR and its expression in plants leads to rapamycin susceptibility. *BMC Plant Biol. 7*, 26−33.
(21) Ren, M., Qiu, S., Venglat, P., Xiang, D., Feng, L., Selvaraj, G., and Datla, D. (2011) Target of Rapamycin Regulates Development and Ribosomal RNA Expression through Kinase Domain in Arabidopsis. *Plant Physiol. 155*, 1367−1382.
(22) Reyes de la Cruz, H., Aguilar, R., and Sanchez de Jimenez, E. (2004) Functional characterization of maize ribosomal S6 protein kinase (ZmS6K), a plants ortholog of methazoan p70 (S6K). *Biochemistry 43*, 533−539.
(23) Berkowitz, O., Jost, R., Pollmann, S., and Masle, J. (2008) Characterization of TCTP the Translationally Controlled Tumor Protein, from *Arabidopsis thaliana*. *Plant Cell 20*, 3430−3447.
(24) Anderson, G. H., and Hanson, M. R. (2005) The *Arabidopsis Mei2* homologue *AML1* binds *AtRaptor1B*, the plant homologue of a major regulator of eukaryotic cell growth. *BMC Plant Biol. 5*, 2.
(25) Moreau, M., Azzopardi, M., Clément, G., Dobrenel, T., Marchive, C., Renne, C., Martin-Magniette, M. L., Taconnat, L., Renou, J. P., Robaglia, C., and Meyer, C. (2012) Mutations in the *Arabidopsis* homolog of LST8/GbL, a partner of the target of rapamycin kinase, impair plant growth, flowering, and metabolic adaptation to long days. *Plant Cell 24*, 463−481.
(26) Ahn, C. S., Han, J. A., Lee, H. S., Lee, S., and Pai, H. S. (2011) The PP2A Regulatory Subunit Tap46, a Component of the TOR Signaling pathway, Modulates Growth and Metabolism in Plants. *Plant Cell 23*, 185−209.
(27) Cohick, W. S., and Clemmons, D. R. (1993) The Insulin-like Growth Factors. *Annu. Rev. Physiol. 55*, 131−153.
(28) Murphy, E., Smith, S., and Smet, I. D. (2012) Small Signaling Peptides in Arabidopsis Development: How Cells Communicate Over a Short Distance. *Plant Cell 24*, 3198−3217.
(29) Matsubayashi, Y., and Sakagami, Y. (2006) Peptide Hormones in Plants. *Annu. Rev. Plant Biol. 57*, 649−674.
(30) Watanabe, Y., Barbashov, S. F., Komatsu, S., Hemmings, A. M., Miyagi, M., Tsunasawa, S., and Hirano, H. (1994) A peptide that stimulates phosphorylation of the plant insulin-binding protein. Isolation, primary structure and cDNA cloning. *Eur. J. Biochem. 224*, 167−172.
(31) Ribeiro, E. D. S., Uchoa, A. F., da Silva, L. G., Beghini, D. G., Ferreira, A. T., Perales, J., Jacinto, T., Fernandez, K. V. S., Xavier-Filho, J., and Oliveira, A. E. A. (2009) Insulin-Binding Canavalia Is Present in *Canavalia ensiformis* seed coat. *Protein Pept. Lett. 16*, 84−91.
(32) García-Flores, C., Aguilar, R., Reyes de la Cruz, H., Albores, M., and Sánchez de Jiménez, E. (2001) A maize insulin-like growth factor signals to a transduction pathway that regulates protein synthesis in maize. *Biochem. J. 358*, 95−100.
(33) Dyer, M. I. (1980) Mammalian epidermal growth factor promotes plant growth. *Proc. Natl. Acad. Sci. U.S.A. 77*, 4836−4837.
(34) Horvath, B. M., Magyar, Z., Zhang, Y. X., Hamburger, A. W., Bako, L., Visser, R. G., Bachem, W., and Bogre, L. (2006) EBP1 regulates organ size through cell growth and proliferation in plants. *EMBO J. 25*, 4909−4920.
(35) Deprost, D., Yao, L., Sormani, R., Moreau, M., Leterreux, G., Nicolai, M., Bedu, M., Robaglia, C., and Meyer, C. (2007) The *Arabidopsis* TOR kinase links plant growth, yield, stress resistance and mRNA translation. *EMBO J. 8*, 864−870.
(36) Rodriguez, C. D., Rodriguez, A., Aguilar, C. R., and Sánchez de Jiménez, E. (2011) Biochemical Characterization of a Maize Novel Peptide Hormone. *Protein Pept. Lett. 18*, 84−91.
(37) Jiménez-López, S., Mancera-Martínez, E., Donayre-Torres, A., Claudia Rangel, C., Uribe, L., March, S., Jiménez-Sánchez, G., and Sánchez de Jiménez, E. (2011) Expression Profile of Maize (*Zea mays* L.) Embryonic Axes During Germination: Translational Regulation of Ribosomal Protein mRNAs. *Plant Cell Physiol. 52* (10), 1719−1733.
(38) Villa−Hernández, J. M., Dinkova, T. D., Aguilar-Caballero, R., Rivera-Cabrera, F., Sánchez de Jiménez, E., and Pérez- Flores, L. J. (2013) Regulation of ribosome biogenesis in maize embryonic axes during germination. *Biochimie 95*, 1871−1879.
(39) Sotelo, R., Garrocho-Villegas, V., Aguilar, C. R., Calderon, M. E., and Sánchez de Jimenez, E. (2010) Coordination of cell growth and cell division in maize (*Zea mays* L.) relevance of the conserved TOR signal transduction pathway. *In Vitro Cell. Dev. Biol.: Plant 46*, 578−586.
(40) Avila-Alejandre, A., Espejel, F., Paz-Lemus, E., Cortés-Barberena, E., Díaz de Leon-Sánchez, F., Dinkova, T. D., Sánchez de Jiménez, E., and Pérez-Flores, L. J. (2013) Effect of insulin on the cell cycle of germinating maize seeds (*Zea mays* L.). *Seed Sci. Res. 23*, 3−14.
(41) Garrocho-Villegas, V., Jesus-Olivera, M. T., and Sánchez, Q. E. (2012) Maize Somatic Embryogenesis: Recent Features to Improve Plant Regeneration. In *Plant Cell Culture Protocols, Methods in Molecular Biology* (Loyola-Vargas, V. M., and Ochoa-Alejo, N., Eds.) Vol. *877*, pp 173−182, Springer, Berlin.

(42) Winegrad, A. I., and Renold, A. E. (1958) Studies on rat adipose tissue in vitro. II. Effects of insulin on the metabolism of specifically labeled glucose. *J. Biol. Chem. 233*, 273−276.
(43) Perez-Mendez, A., Aguilar, R., Briones, E., and Sánchez de Jiménez, E. (1993) Characterization of ribosomal protein phosphorylation in maize axes during germination. *Plant Sci. 94*, 71−79.
(44) Sánchez-Nieto, S., García-Rubio, O., Pacheco-Moisés, F., Carballo, A., Rodríguez-Sotres, R., and Gavilanes-Ruíz, M. (1997) Purification of plasma membranes from dry maize embryos. *Physiol. Plant. 101*, 157−164.
(45) Power, J. B., and Chapman, J. V. (1985) Isolation, culture and genetic manipulation of plant protoplasts. In *Plant Cell Culture, a Practical Approach* (Dixon, R. A., Ed.) pp 37−66, IRL Press, Oxford, U.K.
(46) Fingar, D. C., Richarson, C. J., Tee, A. R., Cheatham, L., Tsou, C., and Blenis, J. (2004) mTOR controls cell cycle progression through its cell growth effectors S6Kl and 4E-BPl/eukaryotic translation initiation factor 4E. *Mol. Cell. Biol. 24*, 200−216.
(47) Guo, Z. Y., Shen, L., and Feng, Y. M. (2002) The Different Folding Behavior of Insulin and Insulin-like Growth Factor 1 Is Mainly Controlled by Their B-Chain/Domain. *Biochemistry 41*, 1556−1567.
(48) Markussen, J., and Volund, A. (1975) Conformational analysis of circular dichroism spectra of insulin, proinsulin and c-peptides by non-linear regression. *Int. J. Pept. Protein Res. 7*, 47−56.
(49) Meyuhas, O. (2000) Synthesis of the translational apparatus is regulated at the translational level. *Eur. J. Biochem. 267*, 6321−6330.
(50) Takano, A., Usui, I., Haruta, T., Kawahara, J., Uno, T., Iwata, M., and Kobayashi, M. (2001) Mammalian target of rapamycin pathway regulates insulin signaling via subcellular redistribution of insulin receptor substrate 1 and integrates nutritional signals and metabolic signals of insulin. *Mol. Cell. Biol. 21*, 5050−5062.
(51) Huang, S., Bjornti, M. A., and Hoghton, P. J. (2003) Rapamycin: Mechanism of action on cellular resistance. *Cancer Biol. Ther. 2*, 222−232.
(52) Thoreen, C. C., Chantranupong, L., Keys, H., Wang, T., Gray, N. S., and Sabatini, D. M. (2012) A unifying model for mTORC1-mediated regulation of mRNA translation. *Nature 485*, 109−113.
(53) Ruvinsky, I., Sharon, N., Lerer, T., Cohen, H., Stolovich-Rain, M., Nir, T., Dor, Y., Zisman, P., and Meyuhas, O. (2005) Ribosomal protein S6/phosphorylation is a determinant of cell size and glucose homeostasis. *Genes Dev. 19*, 2199−2211.
(54) Glendorf, T., Sorensen, R. A., Nishimura, E., Pettersson, I., and Kjeldsen, T. (2008) Importance of Solvent-Exposed Residues of the Insulin B Chain α-helix for Receptor Binding. *Biochemistry 47*, 4743−4751.
(55) Jiráček, J., Žáková, L., Antolíková, E., Watson, J. C., Turkenburg, P. J., Dodson, G. G., and Brzozowski, M. A. (2010) Implications for the active form of human insulin based on the structural convergence of highly active hormone analogues. *Proc. Natl. Acad. Sci. U.S.A. 107* (5), 1966−1970.
(56) Hua, Q. X., Jia, W., and Weiss, M. A. (2011) Conformational Dynamics of Insulin. *Front. Endocriol. 2*, 48.
(57) Chang, J. S., Nakagawa, S., and Steiner, F. D. (2007) Complementation Analysis Demonstrate That Insulin Cross-links Both α Subunits in a Truncated Insulin Receptor Dimer. *J. Biol. Chem. 282*, 13754−13758.
(58) Pache, J. C. (2006) Epidermal Growth Factors. *Encyclopedia of Respiratory Medicine*, 129−133.
(59) Albino, C. L., McNeil, K. A., Ong, S. C., Delaine, C., Booker, G. W., and Wallace, J. (2009) A novel approach to identify two distinct receptor binding surfaces of insulin-like growth factor II. *J. Biol. Chem. 284*, 7656−7664.
(60) He, Z. H., Fujiki, M., and Kohorn, B. D. (1996) A Cell Wall-associated, Receptor-like Protein Kinase. *J. Biol. Chem. 271*, 19789−19793.
(61) Hernández, G., Altmann, M., and Lasko, P. (2009) Origins and evolution of the mechanism regulating translation initiation in eukaryotes. *Trends Biochem. Sci. 35*, 63−73.