Functional Characterization of a Maize Ribosomal S6 Protein Kinase (ZmS6K), a Plant Ortholog of Metazoan p70^{S6K} †

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ABSTRACT: Ribosomal protein S6 (S6rp) is phosphorylated by the p70^{S6K} enzyme in mammals, under mitogen/IGF regulation. This event has been correlated with an increase in 5′TOP mRNA translation. In this research, a maize S6 kinase (ZmS6K) was isolated from maize (*Zea mays* L.) embryonic axes by human p70^{S6K} antibody immunoprecipitation. This enzyme, a 62 kDa peptide, proved to be specific for S6rp phosphorylation, as revealed by *in vivo* and *in vitro* kinase activity using either the 40S ribosomal subunit or the RSK synthetic peptide as the substrates. ZmS6K activation was achieved by phosphorylation on serine/threonine residues. Specific phospho-Threo recognition by the p70^{S6K} antibody directed to target phospho-Threo residue 389 correlated with ZmS6K activation. The ZmS6K protein content remained almost steady during maize seed germination, whereas the ZmS6K activity increased during this process, consistent with Zm6SK phosphorylation. Addition of insulin to germinating maize axes proved to increase ZmS6K activity and the extent of S6rp phosphorylation. These events were blocked by rapamycin, an inhibitor of the insulin signal transduction pathway in mammals, at the TOR (target of rapamycin) enzyme level. We conclude that ZmS6K is a kinase, structurally and functionally ortholog of the mammalian p70^{S6K}, responsible for *in vivo* S6rp phosphorylation in maize. Its activation is induced by insulin in a TOR-dependent manner by phosphorylation on conserved serine/threonine residues.

A substantial proportion of total cellular energy is devoted to the synthesis of new ribosomes during growth and proliferation (*1*). This costly metabolic process must be closely monitored and regulated as a function of nutritional, hormonal, and developmental states (*2*). The mRNAs that encode most ribosomal proteins, termed 5′TOP[1] mRNAs, are characterized by the presence of a 5′-terminal oligopyrimidine track (*3*, *4*). Translation of these messages is selectively stimulated by mitogen/IGF (insulin-like growth factor) and also by amino acids, through a signal transduction pathway, at present not completely understood. Under mitogen/IGF stimulation, phosphorylation of the S6 ribosomal protein (S6rp) on the 40S ribosomal subunit has been elucidated (*5*, *6*). It has also been stated that activation of S6rp kinase (p70^{S6K}) is another pathway target in mammals (*7*, *8*) and in other eukaryotes (S6rpK) (*9*, *10*). This activation depends on the phosphorylation of specific enzyme target sites by a process that involves the TOR (target of rapamycin) kinase (*11*).

In plants, information about this kind of signal transduction pathway is scarce. However, it has been shown that insulin (*12*) or an insulin-related peptide isolated from maize (ZmIGF) (*13*) induces seedling growth, as well as S6rp phosphorylation and ribosomal protein synthesis in germinating embryos, suggesting the presence of a mitogen/IGF-induced pathway in plants, similar to the one regulating cell growth and proliferation in animals.

Despite the fact that S6K has not yet been characterized in plants, reports indicate that S6rp phosphorylation is tightly regulated in these organisms, during physiological (*14*) as well as stress (*15*) conditions. Two clones designated AtS6k1 and AtS6k2 have been isolated from an *Arabidopsis thaliana* cDNA library (*16*). These clones were found to be identical to two previously identified clones termed Atpk1/ATPK6 (*17*, *18*) and ATPK19 (*17*), respectively. Database analysis of AtS6k1 and AtS6k2 showed a high level of conservation of catalytic domains compared with p70^{S6K}. AtS6k2, ectopically expressed in the human 293 cell line, phosphorylated mammalian and plant ribosomal S6 protein *in vitro* and *in vivo* (*16*), demonstrating that AtS6K2 might correspond to the mammalian p70^{S6K}.

Because of the relevance of the mitogen/IGF-induced pathway in regulating growth in a wide range of eukaryotes, and probably also in plants (*13*), further investigations were undertaken. Thus, the objective of this research was to isolate and characterize an S6 protein kinase from germinating maize embryonic axes (ZmS6K) and to determine if this enzyme is functionally equivalent to the mammalian p70^{S6K}. Data presented herein demonstrate that ZmS6K is a TOR-dependent activated kinase, regulated through an insulin/IGF-stimulated signal transduction pathway, similar to that reported in other eukaryotes.

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[1] Abbreviations: S6rp, S6 ribosomal protein; ZmS6K, maize S6 kinase; mTOR, mammalian target of rapamycin; 5′TOP, 5′UTR track of oligopyrimidines.

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MATERIALS AND METHODS

Biological Material. Seeds and embryonic axes of maize (*Zea mays* L. cv. Chalqueño) were used for all experiments. Maize seeds were germinated in water-imbibed cotton at 24 ± 2 °C in darkness. After the stated period (0−48 h), embryonic axes were manually dissected and frozen in liquid nitrogen.

Preparation of Maize Axes Extract. Axes (400 mg) were placed in Murashige-Skoog medium (MS) (*19*) and incubated for the stated period, then frozen in liquid N_2, ground in a mortar, and homogenized in 8 mL of freshly prepared extraction buffer [50 mM HEPES (pH 7.6), 50 mM $Na_4P_2O_7$, 1 mM Na_2VO_4, 1 mM Na_2MoO_4, 4 mM EDTA, 20 mM EGTA, 20 mM NaF, 80 mM β-glycerophosphate, 200 mM mannitol, 2 mM DTT, 0.2 mM PMSF, and 1 mM benzamidine]. The homogenate was centrifuged at 27000*g* and 4 °C for 30 min and the supernatant likewise at 280000*g* for 3.5 h on a sucrose cushion (1.5 M sucrose and 800 mM KCl in extraction buffer). The new supernatant was termed the axes extract.

ZmS6 Kinase Immunoprecipitation. ZmS6K was obtained from the axes extract. Five hundred microliters of axes extract (3 mg of cytoplasmic protein) was diluted in Eppendorf tubes with 500 μL of buffer A [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 0.05% (w/v) Nonidet-P40] with 2 mM benzamidine and 1 μM PMSF, and 5 μL of $p70^{S6K}$ antibody (Santa Cruz Biotechnology catalog no. SC-230) was added to the mixture. The immunoprecipitated mixture was incubated at 4 °C overnight with constant shaking. Then 100 μL of protein A−Sepharose beads (Zymed Laboratories catalog no. 10-1141) was added to the mixture and the mixture incubated at 4 °C for a further 4 h. The immunoprecipitated mixture was passed through a microcolumn using a micropipet tip blocked with glass fiber. The protein A−Sepharose−antibody−protein complex was washed three times with buffer B [100 mM Tris-HCl (pH 7.5), 1 mM NaCl, 0.05% (w/v) Nonidet-P40, and 0.25% (v/w) Triton X-100] and twice with buffer A and eluted with 300 μL of sample buffer [60 mM Tris-NaOH (pH 7.5), 2% (w/v) SDS, and 10% (w/v) glycerol].

40S Ribosomal Subunit Preparation. Maize ribosomes from quiescent seeds were obtained as described previously (*20*) except that the ribosome suspension was layered on a 10 mL hyperbolic sucrose cushion (from 5 to 45% sucrose in extraction buffer) and centrifuged at 19000*g* and 4 °C overnight in a Beckman rotor SW40. The gradient was fractioned, and the fractions were read at 260 nm. The fractions enriched with the 40S ribosomal subunit were centrifuged again at 360000*g* and 4 °C for 2 h; the supernatant was discarded, and the pellet was washed and dissolved with 40 μL of resuspension buffer (*20*). The 40S ribosomal suspension was frozen and thawed three times prior to being used as a kinase substrate.

ZmS6 Kinase Assay and Kinetic Analysis. The protein kinase assay was performed using the immunoprecipitate obtained as described above with some modifications. The protein A−Sepharose−antibody−protein kinase complex was collected at the bottom of Eppendorf tubes by top-speed centrifugation in a microfuge. Then the supernatant was discarded and the pellet washed three times with buffer B (1 mL) and then twice with buffer A. The pellet was resuspended in dilution buffer [50 mM MOPS (pH 7.2), 10 mM *p*-nitrophenyl pyrophosphate, 1 mM DTT, 10 mM $MgCl_2$, and 0.1% (w/v) Triton X-100]. Ten microliters of this complex was used for a routine kinase assay (*21*) in a reaction mixture containing reaction buffer [50 mM MOPS (pH 7.0), 10 mM $MgCl_2$, 1 mM DTT, 10 mM *p*-nitrophenyl pyrophosphate, and PKC and PKA specific inhibitors (500 and 150 ng, respectively)], 100 μM ATP, 5 μCi (3000 Ci/mmol) of [γ-^{32}P]ATP, and either 40 μg of 40S maize ribosomal subunit or 5 nM RSK peptide (Calbiochem Co. catalog no. 559280) in a final volume of 20 μL. The reaction mixture was incubated for 30 min at 24 ± 2 °C, and then the reaction was stopped. (A) With the 40S ribosomal subunit as the substrate, addition of 5 μL of 5× SDS−PAGE sample buffer [0.05 M Tris-HCl (pH 6.8), 0.02 M EDTA, 10% (v/v) glycerol, 1% (w/v) SDS, 1% (w/v) β-mercaptoethanol, and 0.002% (w/v) bromophenol blue], and boiling for 3 min. After electrophoresis, the SDS−PAGE gel was Coomassie stained and dried, and the autoradiography was obtained. (B) With the RSK peptide as the substrate, the reaction was stopped by applying 20 μL of the reaction mixture to p81 phosphocellulose paper circles (Whatman Co. catalog no. 3698323), and then the mixture was extensively washed with 0.85% (v/v) phosphoric acid for 24 h and finally washed with acetone. The papers were dried at room temperature and placed in vials for liquid scintillation counting. For the determination of kinetic parameters, RSK and ATP were used as substrates at different concentrations (0−13.5 nM and 0−500 μM, respectively).

In Vivo ZmS6K and S6rp Phosphorylation. Two sets of maize embryonic axes from seeds germinated for 22 h were dissected and incubated in 1 mL of MS medium for a further 2 h with 400 μCi of [^{32}P]orthophosphate (10 mCi/mL, Amersham Pharmacia Biotech), in the presence or absence of 200 microunits/mL insulin. In some experiments, rapamycin (0.1 μM) was applied 15 min before insulin addition. One set of axes was used to obtain ZmS6K as indicated above. The other set was homogenized in liquid nitrogen with a mortar and pestle and the powder mixed with extraction buffer [20 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 20 mM KCl, 1 mM PMSF, 5 mM NaF, 0.5% 2-mercaptoethanol, 1% Triton X-100, and 0.25 M sucrose]. The mixture was clarified by centrifugation at 27000*g* and 4 °C for 30 min, and the supernatant was layered on a sucrose cushion (0.5 M sucrose and 0.8 M KCl in extraction buffer) and centrifuged at 280000*g* for 3.5 h to obtain the ribosomes. The ribosome pellet was resuspended in buffer [20 mM Hepes (pH 6.5), 20 mM KOH, 5 mM magnesium acetate, 125 mM potassium acetate, and 5% 2-mercaptoethanol], and ribosomal proteins were extracted with acetic acid, precipitated with acetone, and resuspended in water. The ^{32}P-labeled proteins (ribosomal and immunopurified proteins) were analyzed by SDS−PAGE and autoradiography.

Two-Dimensional Electrophoresis Analysis of ZmS6K. Two-dimensional electrophoresis analysis of axes extracts (200 μg of protein) from seeds germinated for 0, 6, 12, 24, and 48 h was performed according to the method of O'Farell (*22*) using a pH gradient from 3.5 to 10 and 12% SDS−polyacrylamide gels. Equal amounts of proteins from the same set of axes were treated with 4 units of alkaline phosphatase (Sigma Chemical Co. catalog no. P-0762). The polyacrylamide gels were transferred to PVDF membranes

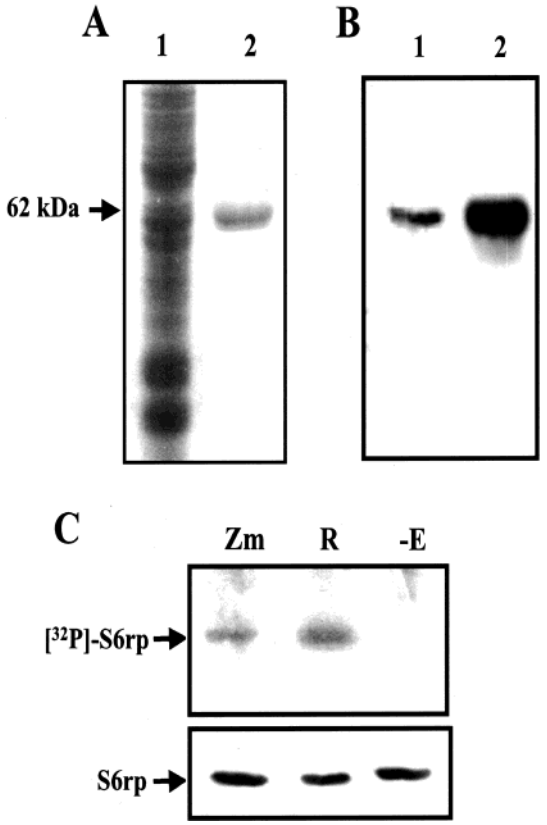

FIGURE 1: Immuno-identification and activity of a maize peptide S6rp*K* (ZmS6K). Maize axes extract from 24 h germinated seeds (lanes 1) or ZmS6K immunoprecipitated from the maize extracts by anti-human p70^{S6K} antibody (lanes 2) were resolved by SDS−PAGE. A) Coomassie stained gel and B) analyzed by Western blot using anti-human p70^{S6K} antibody. The 62 kDa band corresponds to ZmS6K protein. Protein kinase activity was assessed in the 62 kDa maize immunoprecipitated peptide (ZmS6K) by an *in vitro* assay. Substrates used: 5 μCi [^{32}P]-γ-ATP, and maize 40S ribosomal subunit (20 μg) in the assay buffer in a final volume of 20 μL. The reaction was incubated 30 min at 24 °C and the products were analyzed by SDS−PAGE and auto-radiography (C, upper panel). ZmS6K (Zm), rat p70^{S6K} (R) or none (-E) indicates the source of enzyme. The arrow points to [^{32}P]-S6rp (31 kDa). Western blot analysis of a similar gel was performed with S6rp antibody to identify the 31 kDa band (C, lower panel).

(Millipore Co. catalog no. IPVH0010) and assayed by Western blot analysis using anti-human p70^{S6K} (Santa Cruz Biotechnology).

RESULTS

Immunoidentification of a Maize Peptide Recognized by the Anti-Human p70^{S6K} Antibody. To isolate and characterize the maize S6 kinase protein, extracts from germinating maize embryonic axes were immunoprecipitated with the anti-human p70^{S6K} antibody and further purified with protein A−Sepharose and successive buffer detergent washes to eliminate the antibody (Materials and Methods). SDS−PAGE (Figure 1A) and Western blotting (Figure 1B) were performed on both the crude extract and immunoprecipitated protein. A protein with an apparent molecular mass of 62 kDa that cross-reacted with the anti-human p70^{S6K} antibody was observed in both cases. Serum from nonimmunized rabbit and antibody against maize eIF4G were also tested, with negative results (data not shown).

Full-Length ZmS6K cDNA Sequence. The ZmS6K gene sequence was obtained by RT-PCR using maize RNA poly A+ as a template and oligonucleotide primers designed from the most conserved regions of the S6Ks cDNA sequences. The RNA was obtained from embryonic axes dissected from maize seeds germinated for 48 h. This cDNA sequence (NCBI GenBank entry AY389497) was analyzed with the Translate and ScanProsite tools (ExPASy Molecular Biology Server). Results indicate that the ZmS6K cDNA sequence encodes a 488-amino acid protein with a theoretical molecular mass of 54.1 kDa and an isoelectric point of 6.19, compared with the corresponding parameters for p70^{S6K} (56.1 kDa and a pI of 6.02, respectively) (pI/MW tool). The ZmS6K nucleotide sequence predicts a protein that contains a serine/threonine protein kinase signature and that is 48% identical to human p70^{S6K} (NCBI GenBank entry P23443) and 62% identical to *A. thaliana* ATPK6 (NCBI GenBank entry AY096555). Most importantly, the protein conserves two of the main target residues essential for the activation of p70^{S6K} in animals: Thr-389 (Thr-468 in ZmS6K) and Thr-229 (Ser-308 in ZmS6K).

The 62 kDa Protein Has S6 Kinase Activity (ZmS6K). To determine if the 62 kDa protein has S6 kinase activity, an *in vitro* assay system was established. Immunoprecipitated ZmS6K from maize extract germinated for 24 h was used as the enzyme source, and [γ-^{32}P]ATP and the 40S ribosomal subunit were used as substrates (Figure 1C). ZmS6K activity was indicated by the amount of ^{32}P incorporated in S6rp. This was determined by autoradiography of the electrophoresed 40S ribosomal proteins (Figure 1C, Zm). Rat liver p70^{S6K} isolated in the lab by p70^{S6K} antibody immunoprecipitation was used as a positive control of the assay (Figure 1C, R), using also maize 40S subunits as the substrate. A negative control without enzyme was also set (Figure 1C, −E). Results of the autoradiography indicated incorporation of ^{32}P in only a 31 kDa protein from the set of 40S ribosomal subunit proteins, both with maize and rat enzyme but not in the negative control (Figure 1C, top panel). This band was further identified as S6rp by Western blot analysis with the rat S6rp antibody (Figure 1C, bottom panel).

ZmS6K Activity Correlates with Phosphorylation on Equivalent Threo Residue 389. Previous reports have demonstrated that p70^{S6K} kinase activity correlates with the enzyme phosphorylation on Threo residue 389 (*8*, *23*, *24*). To analyze this, the following experiment was set up. Maize axes extracts from different germination periods (from 0 to 48 h) were resolved by SDS−PAGE and analyzed by Western blotting using either anti-human p70^{S6K} to measure total ZmS6K protein content or anti-phospho Threo residue 389 antibodies to test whether the equivalent Threo residue in the ZmS6K protein is phosphorylated. Results indicated that the ZmS6K protein level was similar for all germination periods that were tested (from 0 to 48 h) (Figure 2A, top panel). On the other hand, phosphorylation on equivalent phosphorylated Threo residue 389 was not observed in quiescent axes (0 h) but started to be noticed ~6 h later, and the level of phosphorylation increased toward the end of germination (Figure 2A, bottom panel), suggesting that the activity of this enzyme is developmentally regulated during germination. To confirm that ZmS6K activity correlates with phosphorylation on equivalent Threo residue 389, an *in vitro* assay was set up with the RSK peptide as the substrate, measuring the amount of ^{32}P incorporated into RSK. Different concentrations of the synthetic RSK peptide

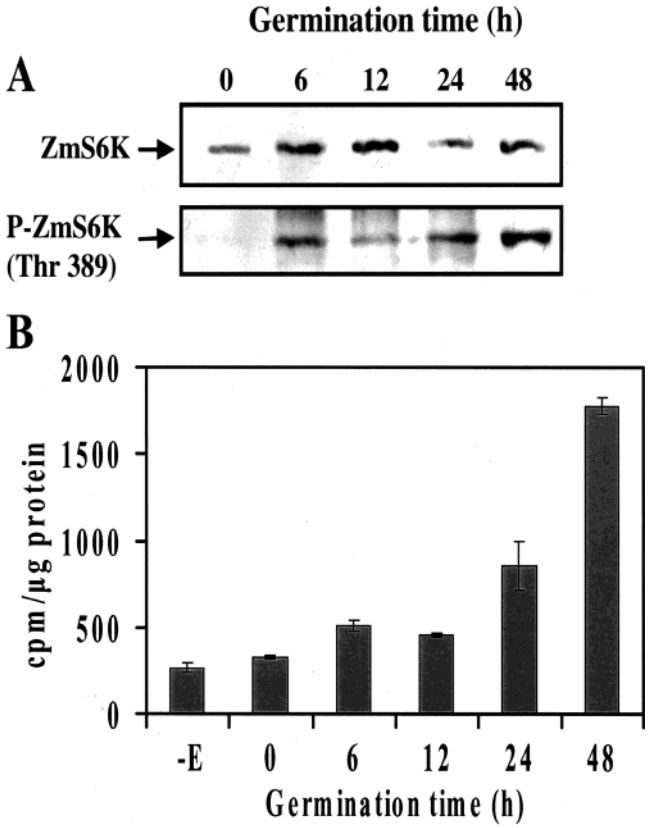

FIGURE 2: ZmS6K expression and activation during germination. Embryonic axes extracts from seeds germinated for 0, 6, 12, 24 and 48 h were SDS−PAGE resolved and analyzed by Western blot either with anti-human $p70^{S6K}$ (top panel) or anti 389 phospho Thr-389 $p70^{S6K}$ (lower panel). B) Immunoprecipitates from the same embryonic axes extracts were analyzed by *in vitro* ZmS6K activity using RSK peptide (5 μg) and [^{32}P]-γ-ATP (5 μCi) as substrates. Results are shown as mean values ± standard error of three independent measurements of the incorporated [^{32}P]. These experiments were reproduced three times with similar results.

and [γ-^{32}P]ATP were tested to define the assay conditions (see Materials and Methods), and the substrate concentrations that were chosen were based on the obtained kinetic parameters. These concentrations turned out to be very close to those reported for the mammalian enzyme (*25*) (data not shown). Thus, the ZmS6 kinase activity was determined in each of the axes extracts from the germination experiment described above. Results showed zero ZmS6K activity in quiescent axes and very low activity after germination for 6 h, slowly increasing toward the end of this period (48 h) (Figure 2B). These data indicate a positive correlation between the phosphorylation status determined by the Threo residue 389 antibody (Figure 2A, bottom panel) and the amount of ^{32}P incorporated *in vitro* into the RSK substrate (Figure 2B), which confirms the dependence of the ZmS6K activity on phosphorylation on equivalent Threo residue 389.

Full activation of the mammalian $p70^{S6K}$ can be reached after multiple phosphorylation steps of different sites besides Threo residue 389 (*8*, *23*, *34*). Thus, the phosphorylation status of the ZmS6K protein during germination was further analyzed. To this end, embryonic axes extracts from germinated seeds at different germination times were resolved by two-dimensional (2D) gel electrophoresis within a pH range of 3.5−10 in the first dimension and analyzed by SDS−PAGE and Western blot with anti-human $p70^{S6K}$ antibody

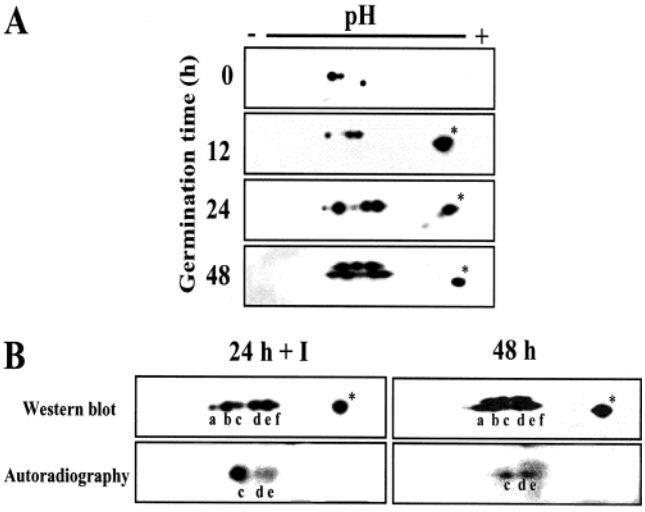

FIGURE 3: 2-D electrophoretic analysis of the S6K phosphorylated forms during germination. A) Crude extracts from embryonic axes germinated for the indicated periods were prepared. Two hundred μg of proteins were analyzed by 2D electrophoresis and Western blot using the anti-human $p70^{S6K}$ antibody. B) Maize embryonic axes from 22 or 46 h germinated seeds were incubated in MS medium for 2 h in the presence of [^{32}P]-orthophosphate. The incubation system for the 22 h axes also contained insulin 200 μU mL^{-1}. Crude extracts from each axes set were prepared and the same amount of proteins as in A) were resolved in the 2D electrophoresis and analyzed by Western blot (upper panels) and autoradiography (lower panels). The asterisk marks a protein that was nonspecifically recognized by the antibody.

(Figure 3). In quiescent axes (0 h), the basal ZmS6K shows two spots. However, as germination progresses, more spots appear toward the acidic pH (five well-defined spots after germination for 24 h and at least eight after 48 h) (Figure 3A), suggesting the appearance of posttranslationally modified ZmS6K forms. To test this possibility, two sets of maize embryonic axes dissected from seeds germinated for either 22 or 46 h were incubated in MS medium in the presence of [^{32}P]orthophosphate for 2 h. During this period, the 22 h axes were stimulated with insulin, which is known to result in S6rp phosphorylation in maize axes (*12*, *13*). Extracts from both sets of axes were resolved by 2D gel electrophoresis and analyzed by autoradiogrphy and Western blotting (Figure 3B). The Western blot revealed five spots in the extract from insulin-stimulated axes (Figure 3B, top left panel) and eight spots in the 48 h axes extract (Figure 3B, top right panel) as observed previously (Figure 3A, 24 and 48 h). On the other hand, the autoradiography showed three spots (Figure 3B, bottom panels, *c*−*e*) in both axes extracts, indicating that spots *a* and *b* correspond to the unphosphorylated ZmS6K whereas ZmS6K spots *c*−*e* are due to phosphorylation. Notice that there is a spot toward the right side of the Western blot (labeled with an asterisk), which seems to be due to unspecific antibody recognition since it is not phosphorylated.

Insulin Stimulates ZmS6K Activity in Germinating Axes. Insulin and insulin-like growth factors (IGFs), widely distributed among eukaryotic organisms (*26*), stimulate a signal transduction pathway that targets $p70^{S6K}$ activation and the subsequent S6rp phosphorylation on the 40S ribosomal subunit (*5*). Because it has been shown that insulin induces maize S6rp phosphorylation in growing tissues (*12*), the insulin effect on ZmS6K activity was measured in maize embryonic axes germinated for 24 h. For these experiments,

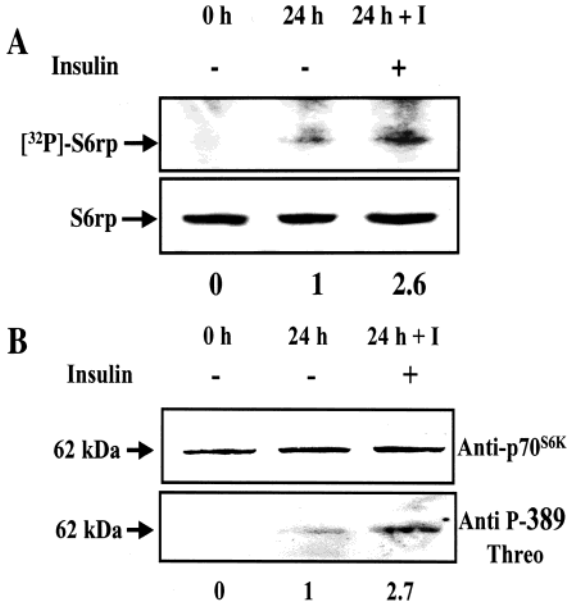

FIGURE 4: Insulin stimulates ZmS6K activity. Maize seeds were 24 h germinated, their axes were dissected and incubated in MS medium without (24 h) or with 200 μUmL^{-1} insulin (24 h + I). Quiescent (nongerminated) axes extract (0 h) was also set as control. ZmS6K was obtained from axes extracts by immunoprecipitation with anti-human p70^{S6K} antibody and ZmS6K activity was *in vitro* assessed using 40S ribosomal subunit as substrate and the same amount of immunoprecipitated ZmS6K from each axes extract. The ribosomal proteins were resolved by SDS−PAGE and autoradiography (A, upper panel) and analyzed by Western blot with anti-S6rp antibody (A, lower panel). Each of the maize extracts from (A) were further analyzed by Western blot either with anti-human p70^{S6K} antibody to assess total amount of ZmS6K protein (B, top panel) or with antibody raised against the target phospho 389-Threo p70^{S6K} region (B, lower panel). The numbers stand for fold of 24 h + I increment as compared with the 24 h control, determined by densitometry.

axes were or were not stimulated with insulin. At the end, ZmS6K was immunoprecipitated from the axes extracts and its activity measured *in vitro* using 40S ribosomal subunits as the substrate and the same amount of ZmS6K protein in each case (Figure 4A). ZmS6K, immunoprecipitated from nongerminating axes (0 h), was also set as a control. Results showed that ZmS6K activity increased nearly 3-fold (2.6) after insulin stimulation with respect to the value of the axes not stimulated for 24 h, as indicated by the intensity of the [^{32}P]S6rp bands resolved on SDS−PAGE and autoradiography (Figure 4A, top panel) and measured by densitometry. On the other hand, the immunoprecipitated ZmS6K from the 0 h extract did not show S6rp phosphorylation**,** indicating the lack of ZmS6K activity in quiescent axes, as shown previously (Figure 2A, bottom panel). Identification of the S6rp in the gels, the endogenous substrate of ZmS6K, was performed by Western blotting with the anti-S6rp antibody (Figure 4A, bottom panel). The endogenous phosphorylation status of ZmS6K in the 24 h nonstimulated and insulin-stimulated maize extracts was further determined. To this end, the same axes extracts from the above experiment were analyzed by Western blotting with both the anti-p70^{S6K} antibody that recognizes the ZmS6K protein (Figure 4B, top panel) and the anti-phospho Threo residue 389 antibody that recognizes only the ZmS6K phosphorylated in the equivalent position (Figure 4B, bottom panel). An increased level of ZmS6K phosphorylation was found in the extract from

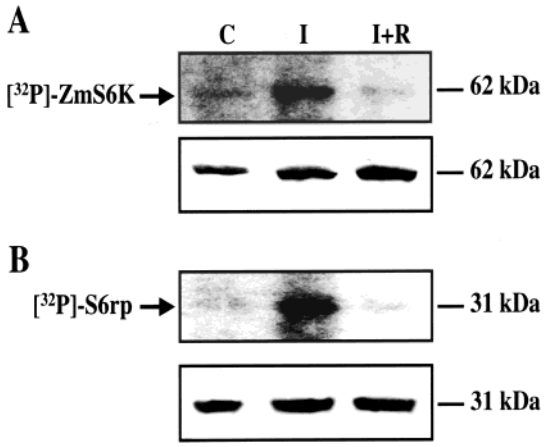

FIGURE 5: Rapamycin inhibition of ZmS6K and S6rp phosphorylation from *in vivo* insulin-stimulated axes. Maize embryonic axes from 22 h germinated seeds were incubated in MS medium with either 200 μUmL^{-1} insulin (I), 200 μUmL^{-1} insulin plus 100 nM rapamycin (I+R) or deionized water (C) and [^{32}P]-orthophosphate (400 μCi) for 2 h more. Autorradiography (A, top panel) and Western blot with p70^{S6K} antibody (A, lower panel) of immunoprecipitated ZmS6K from each axes set. Ribosomal proteins obtained from the same axes set resolved by SDS−PAGE and analyzed by autoradiography (B top panel) and Western blot of these proteins using anti-S6rp antibody (B, lower panel).

insulin-stimulated maize axes (2.7-fold) as compared with phosphorylation of the ZmS6K in the 24 h control (Figure 4B, bottom panel), whereas both extracts exhibited similar amounts of ZmS6K protein (Figure 4B, top panel). The extract from quiescent axes (0 h) did not exhibit reaction with the anti-phospho Threo residue 389 antibody but exhibited almost the same amount of ZmS6K protein by its reaction with the anti-p70^{S6K} antibody. These data indicate that maize ZmS6K is activated after insulin stimulation of maize axes and confirmed that its activity correlates with the level of ZmS6K phosphorylation on the equivalent target threonine residue of the p70^{S6K} enzyme, as reported for the mammalian kinase (*29*).

Effect of Rapamycin on ZmS6K Activity and Phosphorylation. TOR (target of rapamycin) kinase is a main target of the insulin/IGF-stimulated signal transduction pathway that regulates S6K activation in animals (*11*). To test whether ZmS6K activation is also TOR-dependent, an experiment was designed where embryonic axes from seeds germinated for 24 h were insulin-stimulated either alone or in the presence of rapamycin (applied 15 min before the insulin stimulus). This immunosuppressant drug is an inhibitor of the insulin/IGF-stimulated signal transduction pathway at the TOR level (*4*, *27*). A nonstimulated axes group was also set as a control. The axes were fed with [^{32}P]orthophosphate for 2 h as described in Materials and Methods. At the end of this period, half of the axes were used to isolate ribosomal proteins and the other half to immunoprecipitate the ZmS6K. The two groups of proteins were SDS−PAGE resolved and their autoradiographies revealed with a phosphorimager. Results show an increased level of phosphorylation on ZmS6K as well as on S6rp in the insulin-stimulated axes (top panels of parts A and B of Figure 5, respectively), whereas the nonstimulated axes showed low phosphorylation levels in both proteins. The protein load controls in the SDS−PAGE gels showed similar amounts of the tested proteins by Western blotting with the corresponding antibodies

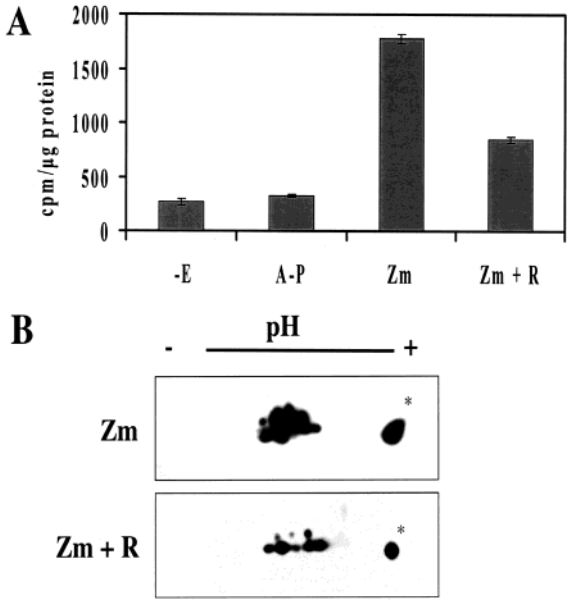

FIGURE 6: Rapamycin effect on endogenous ZmS6K activity from maize. A) *In vitro* ZmS6K activity measurements made with RSK (5 μg) peptide and [^{32}P]-γ-ATP (5 μCi) as substrates in axes extracts from 48 h germinated seeds incubated either with (Zm + R) or without (Zm) rapamycin. Controls correspond to reaction mixture without enzyme (-E) or without substrate (A−P), respectively. B) 2-D electrophoresis and Western blot analysis of axes extracts prepared as in Figure 3 using the same p70^{S6K} antibody.

(bottom panels of parts A and B of Figure 5, respectively). On the other hand, the levels of ZmS6K and S6rp phosphorylation in the axes that were insulin-stimulated in the presence of rapamycin did not show an increment, but their levels were similar to the control (top panels of parts A and B of Figure 5). These data indicate that rapamycin blocked the insulin stimulatory effect on ZmS6K and as a consequence on S6rp. The positive correlation observed between *in vivo* ZmS6K activation and S6rp phosphorylation indicates that ZmS6K is the enzyme responsible for *in vivo* S6rp phosphorylation, and also that a similar TOR kinase may lay upstream of the ZmS6K within the corresponding signal transduction pathway in maize.

Further, the effect of rapamycin on ZmS6K endogenous activity was analyzed. Axes from seeds germinated for 48 h, where ZmS6K is already fully active (Figure 2), were incubated with or without rapamycin under similar conditions as described above. ZmS6K was immunoprecipitated from the two sets of 48 h axes and its activity measured *in vitro* with RSK and [γ-^{32}P]ATP. As seen in Figure 6A, rapamycin inhibited ZmS6K endogenous activity, to a significant extent (more than half) compared with the enzyme from axes not incubated with rapamycin (Figure 6A). A control without RSK substrate was also set to test whether ZmS6K is capable of autophosphorylation. Figure 6A (A-P) shows that this is not the case.

To complete these data, analysis of the phosphorylated status of the 48 h axes ZmS6K, with or without rapamycin, was performed by isoelectrofocusing and Western blotting on maize extracts from axes germinated for 48 h with the anti-human p70^{S6K} antibody. Results showed that the samples not treated with rapamycin for 48 h presented at least eight strong spots within the acidic pH range (Figure 6B, top panel). With rapamycin present, however, many of these spots were not present in the pattern, and the intensity of the most acidic spots decreased relative to the control (Figure 6, bottom panel). From this result, it can be concluded that rapamycin inhibits ZmS6K endogenous activity as well.

DISCUSSION

This research characterizes, for the first time in plants, the role of a protein kinase (ZmS6K) specific for S6rp phosphorylation in maize ribosomes. cDNAs encoding for putative S6Ks have, however, been reported previously for *Arabidopsis*, where two cDNAs were found, namely Atp*K*1/ATPK6 and Atp*K*2/ATPK19 (*16*, *17*, *18*). For maize, our data indicate one main active ZmS6K multiphosphorylated protein, as judged by the higher mobility toward the acidic IEF pH region observed after enzyme activation (Figures 3 and 6B), coincident with the [^{32}P] incorporation found on the most acidic spots (Figure 3B, lower panels). Furthermore, this spots where not present when maize extract were alkaline phosphatase treated (data not shown). All these data are consistent with the seven putative phosphorylation sites predicted by the ScanProsite analysis of the ZmS6K cDNA sequence. This analysis also indicates four N-glycosilation and four N-myristoylation sites, suggesting that other ZmS6K posttranslational modifications, besides phosphorylation, might account for the spots that migrate above the normal ZmS6K molecular mass (Figure 3, 48 h and Figure 6B).

The 62kDa maize ZmS6K presents several similarities with the correspondent mammalian enzyme. Indeed, ZmS6K and p70^{S6K} proteins share structural and functional characteristics, such as specific recognition by the same anti-human p70^{S6K} antibody (Figure 1B), and substrate specificity: the endogenous S6rp on the 40S ribosomal subunit (Figs. 1C, and 4A) and the synthetic RSK peptide (Figs. 2B and 6A) (*8*, *28*). Furthermore, both enzymes seem to have a similar activation mechanism. ZmS6K showed several phosphorylated sites in the active enzyme (Figures 2 and 3) that positively correlates with its activity and the phosphorylation on a target site recognized by the 389-Threo specific antibody residue (Figures 2 and 4 lower panels), correspondent with p70^{S6K} activation (*8*, *23*, *24*, *29*). Moreover, both enzymes proved to be highly sensitive to rapamycin inhibition indicating its dependency on TOR kinase activity, an enzyme located upstream p70^{S6K} within the insulin/IGF signal transduction pathway (Figures 5 and 6) (*11*). All these data clearly indicate that ZmS6K is the ortholog maize enzyme of the human p70^{S6K}.

Several studies have shown that the S6K signaling pathway induced by mitogens/IGFs plays a critical role in animal cell growth (*10*, *30*, *31*). This pathway is known to induce the activation of the rapamycin-sensitive mTOR and p70^{S6K} enzymes, and correlates with the increase of specific translation of the 5′TOP mRNA messengers (*32*), that results in the regulation of cell growth and proliferation (*33*, *34*). In maize, an insulin-related growth factor (ZmIGF), with structural and functional similarities to insulin, has been isolated and purified from maize embryonic axes (*13*). Moreover, ZmIGF as well as insulin have proved to stimulate S6rp phosphorylation and ribosomal protein synthesis in maize axes, as well as to accelerate seed germination and seedling growth (*12*, *13*). These data strongly suggest that ZmIGF is the endogenous effector of a signal transduction pathway that regulates cell growth and proliferation in maize.

This interpretation is further supported by the results from the insulin-stimulated axes experiments, showing significant *in vivo* and *in vitro* increments on ZmS6K activity in a rapamycin-sensitive manner (Figs. 4A and 5).

The above data and the recent finding of a TOR gene in *A. thaliana* (*35*), showing high level of identity with the human mTOR gene (*36*, *37*), strengthen our interpretation that maize, and probably plants in general, regulate cell growth and proliferation through a similar pathway as animal eukaryotes.

In summary a maize protein kinase, ZmS6K, structurally and functionally ortholog to the mammalian $p70^{S6K}$ is reported. ZmS6K showed to be specifically activated by phosphorylation, subject to regulation by insulin stimulation and inhibited by rapamycin. This information strongly suggests that this enzyme is part of an endogenous insulin-like growth factor (ZmIGF)-stimulated signal transduction pathway that regulates cell growth and proliferation in maize tissues.

REFERENCES

1. Warner, J. R. (1999) The economics of ribosomal biosynthesis in yeast, *Trends Biochem. Sci. 24*, 437−440.
2. Rutter, J, Probst, B. L., and McKnight, S. L. (2002) Coordinate regulation of sugar flux and translation by PAS kinase. *Cell 111*, 17−28.
3. Meyuhas, O., and Horstein, E. (2000) Translational control of TOP mRNAs, in *Translational Control of Gene Expression* (Hershey, J. W. B., Mathews, M. B., Sonenberg, N., Ed.) pp 671−695, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
4. Grolleau, A., Bowman, J., Pradet-Balade, B., Puravs, E., Hanash, S., Garcia-Sanz J. A., and Beretta, L. (2002) Global and specific translational control by rapamycin in T cells uncovered by microarrays and proteomics, *J. Biol. Chem. 277*, 22175−22184.
5. Fumagalli, S., and Thomas, G. (2000) S6 Phosphorylation and signal transduction, in *Translational Control of Gene Expression* (Hershey, J. W. B., Mathews, M. B., Sonenberg, N., Ed.) pp 695−717, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
6. Kimball, S. R., and Jefferson, L. S. (2002) Control of protein synthesis by amino acid availability, *Curr. Opin. Clin. Nutr. Metab. Care 5*, 63−67.
7. Stewart, M. J., and Thomas, G. (1994) Mitogen and protein synthesis: A role for ribosomal protein S6 phosphorylation? *BioEssays 16*, 809−815.
8. Law, B. K., Waltner-Law, M. E., Entingh, A. J., Chytil, A., Aakre, M. E., Nørgaard, P., and Moses, H. L. (2000). Salicylate-induced growth arrest is associated with inhibition of $p70^{s6k}$ and down-regulation of c-Myc, ciclin D1, ciclin A, and proliferating cell nuclear antigen, *J. Biol. Chem. 275*, 38261−38267.
9. Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidayat, S., Tokunaga, C., Avruch, J., and Yonezawa, K. (2002) Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action, *Cell 110*, 177−189.
10. Montagne, J., Stewart, M. J., Stocker, H., Hafen, E., Kozma, S. C., and Thomas, G. (1999) *Drosophila* S6 kinase: A regulator of cell size, *Science 285*, 2126−2129.
11. Schmelzle, T., and Hall, M. N. (2000) TOR, a central controller of cell growth, *Cell 103*, 253−262.
12. Sánchez de Jiménez, E., Beltrán-Peña, E., and Ortiz-López, A. (1999) Insulin-stimulated ribosomal protein synthesis in maize embryonic axes during germination, *Physiol. Plant. 105*, 148−155.
13. García-Flores, C., Aguilar, R., Reyes de la Cruz, H., Albores, M., and Sánchez de Jiménz, E. (2001) A maize insulin-like growth factor signals to a transduction pathway that regulates protein synthesis in maize, *Biochem. J. 358*, 95−100.
14. Pérez-Méndez, 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.
15. Scharf, K. D., and Nover, L. (1982) Heat-shock induced alterations of ribosomal protein phosphorylation in plant cell cultures, *Cell 30*, 427−437.
16. Turck, F., Kozma, S. C., Thomas, G., and Nagy, F. (1998) A heat-sensitive *Arabidopsis thaliana* kinase substitutes for human $p70^{S6k}$ function in vivo, *Mol. Cell. Biol. 18*, 2038−2044.
17. Mizoguchi, T., Hayashida, N., Yamaguchi-Shinozaki, K., Kamada, H., and Shinozaki, K. (1995) Two genes that encode ribosomal-protein S6 kinase homologs are induced by cold or salinity stress in *Arabidopsis thaliana*, *FEBS Lett. 358*, 199−204.
18. Zhang, S.-H., Broome, M. A., Lawton, M. A., Hunter, T., and Lamb, C. J. (1994) *atpk1*, a novel ribosomal protein kinase gene from Arabidopsis. II Functional and biochemical analysis of encoded protein, *J. Biol. Chem. 269*, 17593−17599.
19. Murashige, T., and Skoog, C. (1962) A revised medium for rapid growth and bioassay with tobacco cultures, *Physiol. Plant. 15*, 473−479.
20. Beltrán-Peña, E., Aguilar, R., Ortiz-López, A., Dinkova, T. D., and Sánchez de Jiménez, E. (2002) Auxin stimulates S6 ribosomal protein phosphorylation in maize thereby affecting protein synthesis regulation, *Physiol. Plant. 115*, 291−297.
21. Lane, H. A., and Thomas, G. (1991) Purification and properties of mitogen-activated S6 kinase fron rat liver and 3T3 cells, *Methods Enzymol. 200*, 268−291.
22. O′Farell, P. H. (1975) High resolution of two-dimensional electrophoresis of proteins, *J. Biol. Chem. 250*, 4007−4021.
23. Belham, C., Comb, M. J., and Avruch, J. (2001) Identification of the NIMA family kinases NEK6/7 as regulators of the p70 ribosomal S6 kinase, *Curr. Biol. 11*, 1155−1167.
24. Zhang, Y., Dong, Z., Namura, M., Zhong, S., Chen, N., Bode, A. M., and Dong, Z. (2001) Signal transduction pathways involved in phosphorylation and activation of p70S6K following exposure to UVA irradiation, *J. Biol. Chem. 276*, 20913−20923.
25. Law, B. K., Nørgaard, P., Gnudi, L., Kahn, B. B., Poulson, H. S., and Moses H. L. (1999) Inhibition of DNA synthesis by a farnesyltransferase inhibitor involves inhibition of the $p70^{s6k}$ pathway, *J. Boil. Chem. 274*, 4743−4748.
26. White, M. F. (1998) The IRS-signaling system: a network of docking proteins that mediate insulin action, *Mol. Cell. Biochem. 182*, 3−11.
27. Brown, E. J., Albers, M. W., Shin, T. B., Ichikawa, K., Keith, C. T., Lane, W. S., and Schreiber, S. L. (1994) A mammalian protein targeted by G1-arresting rapamycin-receptor complex, *Nature 369*, 756−758.
28. Shah, 0. J., Kimball, S. R., and Jefferson, S. (2000) Among translatioal effectors, $p70^{S6k}$ is uniquely sensitive to inhibition by glucocorticoids, *Biochem. J. 347*, 389−397.
29. Dennis, P. B., Pullen, N., Kozma, S. C., and Thomas, G. (1996) The principal rapamycin-sensitive $p70^{S6K}$ phosphorylation sites, T-229 and T-389, are differentially regulated by rapamycin-insensitive kinase kinases, *Moll. Cell. Biol. 16*, 6242−6251.
30. Shima, H., Pende, M., Chen, Y., Fumagalli, S., Thomas, G., and Kosma, S. C. (1998) Disruption of the p70s6k/p85s6k gene reveals a small mouse phenotype and a new functional S6 kinase, *ENBO J. 17*, 6649−6659.
31. Thomas, G. (2002) The S6 kinase signaling pathway in the control of development and growth, *Biol. Res. 35*, 305−513.
32. 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.
33. Zhang, H., Stallock, J. P., Ng, J. C., Reinhard, C., and Neufeld, T. P. (2000) Regulation of cellular growth by the Drosophila target of rapamycin dTOR, *Genes Dev. 14*, 2712−2724.
34. Proud, C. G. (2002) Regulation of mammalian translation factors by nutrients, *Eur. J. Biochem. 269*, 5338−5349.
35. Menand, B., Desnos, T., Nussaume, L., Berger, F., Bouchez, D., Meyer, C., and Robaglia, C. (2002) Expression and disruption of the *Arabidopsis* TOR (target of rapamycin) gene, *Proc. Natl. Acad. Sci. U.S.A. 99*, 6422−6427.
36. Scott, P. H., Brunn, G. J., Kohn, A. D., Roth, R. A., and Lawrence, J. C., Jr. (1998) Evidence of insulin-stimulated phosphorylation and activation of the mammalian target of rapamycin mediated by protein kinase B signaling pathway, *Proc. Natl. Acad. Sci. U.S.A. 95*, 7772−7777.
37. Huffman, T. A., Mothe-Satney, I., and Lawrence, J. C., Jr. (2002) Insulin-stimulated phosphorylation of lipin mediated by the mammalian target of rapamycin, *Proc. Natl. Acad. Sci. U.S.A. 99*, 1047−1052.

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