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FUNCTIONAL ANALYSIS OF THE PP2A SUBFAMILY OF PROTEIN PHOSPHATASES IN REGULATING DROSOPHILA S6 KINASE

Vincent A. Bielinski and Marc C. Mumby

Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX, 75390-9041

Abstract

Phosphorylation and activation of ribosomal S6 protein kinase is an important link in the regulation of cell size by the target of rapamycin (TOR) protein kinase. A combination of selective inhibition and RNA interference were used to test the roles of members of the PP2A subfamily of protein phosphatases in dephosphorylation of *Drosophila* S6 kinase (dS6K). Treatment of *Drosophila* Schneider 2 cells with calyculin A, a selective inhibitor of PP2A-like phosphatases, resulted in a 7-fold increase in the basal level of dS6K phosphorylation at the TOR phosphorylation site (Thr398) and blocked dephosphorylation following inactivation of TOR by amino acid starvation or rapamycin treatment. Knockdown of the PP2A catalytic subunit increased basal dS6K phosphorylation and inhibited dephosphorylation induced by amino acid withdrawal. In contrast, depletion of the catalytic subunits of the other two members of the subfamily did not enhance dS6K phosphorylation. Knockdown of PP4 caused a 20% decrease in dS6K phosphorylation and knockdown of PP6 had no effect. Knockdown of the *Drosophila* B56-2 subunit resulted in enhanced dephosphorylation of dS6K following removal of amino acids. In contrast, knockdown of the homologs of the other PP2A regulatory subunits had no effects. Knockdown of the *Drosophila* homolog of the PP2A/PP4/PP6 interaction protein α /Tap42 did not affect S6K phosphorylation, but did induce apoptosis. These results indicate that PP2A, but not other members of this subfamily, is likely to be a major S6K phosphatase in intact cells and is consistent with an important role for this phosphatase in the TOR pathway.

INTRODUCTION

The target of rapamycin (TOR) is a conserved protein kinase that lies at the hub of a signaling network responsible for sensing and integrating nutritional status, growth stimuli, and cell stress. Two of the best characterized targets of mammalian TOR, the ribosomal S6 protein kinases (S6K) and the eukaryotic initiation factor 4E binding proteins, are regulators of protein synthesis [1–3]. TOR-dependent activation of mammalian S6K increases protein synthesis by promoting assembly of the eukaryotic translation preinitiation complex [4]. S6K is encoded by two genes in mammals, S6K1 and S6K2. Genetic experiments in mice showed that S6K1 is an essential mediator of the effects of TOR signaling on cell size and mass [5]. The single *Drosophila* homolog of S6K (dS6K) also plays a critical role in cell growth as flies lacking the gene have a delay in development, lower body weight, and smaller cells than wild-type flies [6].

Address correspondence to: Marc Mumby, Ph.D., Dept. of Pharmacology, Univ. of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9041, 214-645-6152; 214-645-6151 FAX marc.mumby@utsouthwestern.edu.

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Activation of mammalian S6K1 involves phosphorylation at multiple sites in response to nutrients and growth factors. Activation of S6K is initiated by phosphorylation of Thr389 within its hydrophobic motif by the TOR/raptor complex. Phosphorylation of Thr389 generates a docking site for phosphoinositide-dependent kinase 1 (PDK1), which phosphorylates Thr229 within the activation loop leading to activation of kinase activity. Inhibition of TOR activity with rapamycin leads to rapid dephosphorylation of these two sites [7]. The TOR and PDK1 phosphorylation sites (Thr238 and Thr398 in dS6K) are conserved in *Drosophila* S6K. S6K phosphorylation at Thr398 by *Drosophila* TOR (dTOR) is essential for kinase activation [8].

The protein phosphatases involved in the physiological dephosphorylation and inactivation of S6K have not been identified. Both mammalian S6K and dS6K are inactivated by dephosphorylation of the TOR site following amino acid starvation or inhibition of TOR with rapamycin [8,9]. An initial link between the PP2A subfamily of protein phosphatases and TOR came from genetic experiments in yeast [reviewed in 10]. The phosphatase 2A associated protein of 42-kDa (Tap42) associates with the catalytic subunits of the yeast PP2A subfamily (PP2A, PP4, and PP6). Tap42 is a major effector of TOR signaling in yeast that interacts with the PP2A subfamily following phosphorylation by TOR [11]. Mammalian cells express a protein (α 4/mTap42) that has 23% amino acid sequence identity with yeast Tap42. The α 4/mTap42 protein interacts directly with the catalytic subunits of PP2A, PP4 and PP6 [12–15] and modulates their enzyme activity [16]. The α 4/mTap42 protein has also been reported to interact with mammalian S6K [17]. Although interaction of Tap42 with the PP2A subfamily has been conserved in higher eukaryotes, a role in TOR signaling has not been established. Unlike yeast Tap42, disruption of *Drosophila* Tap42 has no effect on cell growth [18].

Biochemical studies also support a role for PP2A subfamily phosphatases in TOR signaling in mammalian cells. *In vitro* fractionation and enzymatic characterization indicate that S6K is dephosphorylated by PP2A [19]. The catalytic subunit of PP2A can be isolated in a complex with S6K following cross-linking of soluble brain extracts [20] or by immunoprecipitation of S6K from Jurkat T cell lysates [21]. Dephosphorylation of S6K following amino acid starvation, rapamycin treatment, or cell stress is blocked by inhibitors with selectivity for the PP2A subfamily [21,22]. However, these inhibitors cannot distinguish between the PP2A-like phosphatases [16,23]. Consequently, the phosphatase that dephosphorylates S6K *in vivo* has not been identified.

PP2A, PP4, and PP6 comprise the type 2A subfamily within the serine/threonine phosphatase gene family. These enzymes play vital roles in multiple aspects of cellular signal transduction [24]. The term PP2A refers to a diverse group of phosphatases composed of a common catalytic subunit that forms oligomeric complexes with a variety of regulatory proteins. The most prevalent forms of PP2A contain a core dimer composed of the catalytic subunit and a scaffold protein termed the A subunit. The core dimer binds additional regulatory subunits that target PP2A to specific substrates [25,26]. The mammalian PP4 catalytic subunit also interacts with scaffold and regulatory subunits [27,28], while PP6 regulatory proteins have been identified in yeast [29]. Due to decreased complexity relative to mammalian cells, *Drosophila* has been a valuable system to study functions of individual catalytic and regulatory subunits within the PP2A subfamily [30–32]. The *Drosophila* genome contains homologs of the PP2A catalytic, scaffold, and regulatory subunits [30,31], as well as homologs of PP4 [33], PP6 [34], and Tap42 [18].

To determine if one or more members of the PP2A subfamily or its regulatory proteins regulate S6K *in vivo*, a combined approach using pharmacological inhibition and RNA interference in *Drosophila* cells was used to assess their roles controlling S6K phosphorylation at the TOR phosphorylation site. The results show that PP2A plays an important role in dephosphorylation

of dS6K, while PP4, PP6, and dTap42 are not directly involved in regulating phosphorylation of this site.

MATERIALS AND METHODS

Antibodies

A mouse monoclonal antibody specific for the S6K phosphorylated on Thr389 of the mammalian protein or Thr398 of *Drosophila* S6K was purchased from Cell Signaling Technology. A rabbit polyclonal antibody directed against the C-terminus (residues 435–490) of *Drosophila* S6K [35] was a generous gift from Dr. Mary Stewart. Antibody against the catalytic subunit of PP2A (1D6) was purchased from Upstate Biotechnology. Rabbit antibodies that recognize the A (F725) and the B56-1 (M878) subunits of *Drosophila* PP2A have been described [31]. Horse radish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from GE Healthscience. Anti-mouse Alexafluor 467-conjugated secondary antibody was purchased from Molecular Probes.

Production of Double-Stranded RNA

Complimentary DNA clones corresponding to *Drosophila* PP2A subunits and PP4 have been described [31,32]. A plasmid containing the full-length sequence of *Drosophila* PP6 (*SD01279*) was obtained from the *Drosophila* Genomics Resource Center. A full-length *Drosophila* Tap42 (*dTap42/CG31852*) cDNA was generated by PCR from a 4–8 hour *Drosophila* embryo cDNA library (a gift of Dr. Gregory Tall) using the following primers; 5'-*ATGGCTGAGGGTAATACCGCTGG*-3' (sense strand) and 5'-*GACGGCAACCGTCATAACCGTAGTTAA*-3' (anti-sense strand). Double-stranded RNA was produced from PCR fragments generated from these templates as described [31,32]. The nucleotide sequences of primers used for individual PCR reactions are listed in Table 1.

Cell Culture and RNA interference

Drosophila Schneider 2 cells were maintained in Schneider's *Drosophila* medium [36] without peptone and supplemented with 10% fetal bovine serum at 25°C. RNA interference was carried out as described [37] except that double-stranded RNA (15 µg) was added at 24, 48 and 72 hours after plating the cells. Seventy-two hours after initiating dsRNA treatment, the cells were treated with rapamycin or starved for amino acids as described below.

Cell treatment and lysis

To determine the effects of inhibiting PP2A-like phosphatases on dS6K, S2 cells that had not been treated with dsRNA, were incubated for 15 minutes at 25°C with 50 nM calyculin A (Cell Signaling Technology). Rapamycin was then added to a final concentration of 20 nM or the cells were resuspended in amino acid-free medium containing 50 nM calyculin A. Samples were collected at the time points indicated in the figures and analyzed for S6K phosphorylation.

To determine the effects of depleting phosphatase subunits on dS6K phosphorylation, S2 cells were treated with dsRNA for 72 hours as described above. The effects of dsRNA treatment on rapamycin-induced dephosphorylation of dS6K were determined by incubating the cells with 20 nM rapamycin as described above. To determine dsRNA effects on dS6K dephosphorylation induced by amino acid starvation, the cells were detached from the culture plates by repeated pipetting and 1.5×10^7 cells transferred to a 50 ml conical tube. The cells were collected by centrifugation for 5 minutes at $2,800 \times g$ at 25°C. The medium was aspirated and the cells resuspended in 3 ml serum-free medium at a density of 5×10^6 cells/ml. The cells were allowed to recover for 30 minutes at room temperature. Initial samples (time 0) were collected by transferring a 0.25 ml aliquot (1.3×10^6 cells) of the cell suspension to a 1.5 ml

tube followed by centrifugation at $14,000 \times g$ for 30 seconds. The medium was aspirated and cells lysed in 0.1 ml of 5% SDS sample buffer (0.25M Tris-HCl, pH 6.7; 10% glycerol; 5% sodium dodecyl sulfate; 0.1% bromphenol blue; 2% β -mercaptoethanol). Amino acid starvation was begun by transferring 2 ml of the remaining cell suspension to a 2 ml tube and collecting the cells by centrifugation at $14,000 \times g$ for 30 seconds. The medium was aspirated and the cells resuspended in 2 ml of medium lacking amino acids (final density of 5×10^6 cells/ml). The cells were incubated at room temperature and samples (0.125 ml) collected at the time points indicated in the figures. The cells were collected by centrifugation and lysed in 50 μ l 5% SDS sample buffer. Samples were heated for 3 minutes at 95°C , vortexed for 30 seconds to shear genomic DNA, centrifuged for 10 minutes at $14,000 \times g$, and stored at -20°C . In some experiments, the dsRNA treatments and cell incubations were conducted in the presence of the caspase inhibitor Z-VAD-fmk (30 μ M) to inhibit apoptosis as described [32].

Immunoblotting and quantitation of S6K phosphorylation

Whole cell lysates were probed for total dS6K, phospho-Thr398 S6K, the catalytic and B56-1 subunits of PP2A, and β -actin by immunoblotting following SDS polyacrylamide electrophoresis as described [31]. Bound antibodies were detected by incubating with horse radish peroxidase-conjugated secondary antibodies and visualized by incubating the transferred membranes with ECL reagent (GE Healthcare) and exposure to X-ray film.

For quantitative immunoblotting of dS6K phosphorylation, the transferred membranes were simultaneously incubated with a mouse monoclonal antibody against phospho-Thr398 S6K and a rabbit antibody against total *Drosophila* S6K. The membranes were then incubated with a mixture of horse radish peroxidase-conjugated anti-rabbit and Alexafluor 467-conjugated antimouse secondary antibodies. The membranes were then incubated with ECL-Plus reagent (GE Healthcare) and the signals detected by scanning the membranes with a Molecular Dynamics Storm 860 fluorescence scanner. The Alexafluor 467 signal from the anti-mouse antibody was detected by scanning at a wavelength of 540 nm and the anti-rabbit secondary antibody visualized by detection of the fluorescent intermediate generated from the ECL Plus reaction by scanning at 467 nm. Quantitation of immunoreactive band intensities was carried out using Image Master (GE Healthcare). In experiments where the total S6K signal was resolved into multiple bands, all of the immunoreactive species were included in the quantitation. The ratio of phospho-S6K intensity to total S6K intensity was calculated for each sample and the fold-change caused by the various treatments calculated by dividing the phospho-S6K:total S6K ratio of the experimental samples by the phospho-S6K:total S6K ratio of untreated control samples. The data are expressed as the mean $\pm$ standard deviation of three or four separate experiments.

RT-PCR and real time quantitative PCR

RNAi-mediated knockdown was confirmed by RT-PCR for those phosphatase subunits for which an antibody against the *Drosophila* protein was not available. Total RNA was isolated using the Tripure isolation reagent (Roche) followed by RT-PCR with subunit-specific primers using the Superscript One-Step RT-PCR kit (Invitrogen) as described [38]. The integrity of the RNA used was verified in PCR reactions using primers for the *stubarista* gene (Table 1) whose mRNA levels are not affected by knockdown of PP2A subunits [32]. Knockdown of *Drosophila* Tap42 was confirmed using quantitative real-time PCR. Quantitative PCR was carried out using the following dTap42-specific primers; 5'-AGTTGATCGACGAGGTGTCC-3' (sense strand) and 5'-GGTGGACGATGCAGATT-3' (anti-sense strand). A primer set against *Drosophila* actin was utilized as a control for determination of ΔCt and the fold change between control and dsRNA-treated cells calculated using the $\Delta\Delta\text{Ct}$ method as described [32].

RESULTS

Drosophila S6 Kinase is Dephosphorylated by a Calyculin A-sensitive Phosphatase

The phosphatase inhibitor calyculin A was used to determine if a PP2A subfamily phosphatase was involved in regulating *Drosophila* S6 kinase. Calyculin A is 7- to 9-fold more potent toward PP2A than PP1 *in vitro* [39] and, when used at concentrations of 50 nM or less, is selective for inhibition of the PP2A subfamily of protein phosphatases in intact cells [40,41]. S2 cells were treated with 50 nM calyculin A for 15 minutes and then starved for amino acids or treated with 20 nM rapamycin to inhibit TOR activity. Treatment with calyculin A caused 7-fold increase in the basal level of S6 kinase phosphorylation at Thr398 compared to cells treated with vehicle alone (Fig. 1). Removal of amino acids caused a dephosphorylation of S6 kinase that was completely blocked by calyculin A. The rapid dephosphorylation of S6 kinase caused by treating cells with rapamycin was also blocked by calyculin A. These results show that dephosphorylation of *Drosophila* S6 kinase in response to amino acid starvation or rapamycin treatment is likely to be mediated by a member of the type 2A subfamily of serine/threonine protein phosphatases.

Knockdown of the PP2A Catalytic Subunit Enhances Phosphorylation of Drosophila S6 Kinase

The experiments with calyculin A indicated that a PP2A-type phosphatase was involved in dephosphorylation of S6 kinase in *Drosophila* S2 cells. In order to determine which members of the PP2A subfamily play roles in regulating S6 kinase, the catalytic subunits of each enzyme were knocked down using RNA interference. S2 cells were treated with dsRNA targeting the PP2A catalytic subunit or a control dsRNA corresponding to green fluorescent protein. The efficiency of PP2A catalytic subunit knockdown was determined by immunoblotting and ranged from 70–80% (Fig. 2B, inset). Following knockdown of the PP2A catalytic subunit, cells were starved for amino acids or treated with rapamycin and phosphorylation of Thr398 of dS6K was monitored by Western blotting. Depletion of the PP2A catalytic subunit resulted in a 1.6-fold increase in basal S6K phosphorylation relative to cells that were not treated with dsRNA (Fig. 2). Treatment with the control EGFP dsRNA had no effect on basal Thr398 phosphorylation. Twenty minutes after removal of amino acids, dS6K in control dsRNA cells was dephosphorylated to a plateau level of phosphorylation that was 30% of that in untreated cells. While dS6K was also dephosphorylated following removal of amino acids from cells treated with the PP2A catalytic subunit dsRNA, the extent of dephosphorylation was much lower than in control cells. The plateau level of phosphorylation in cells depleted of the catalytic subunit was the same as that in untreated cells maintained in complete medium. After 60 minutes of amino acid starvation, the level of S6K phosphorylation was 3-fold higher in cells depleted of the PP2A catalytic subunit than in cells treated with control dsRNA. The phosphorylation of S6K was significantly higher ($p<0.05$) in catalytic subunit depleted cells at each time point tested (Fig. 2B).

The effect of depleting the PP2A catalytic subunit on S6K phosphorylation was also determined following inhibition of dTOR with rapamycin. Rapamycin treatment caused a rapid dephosphorylation of S6K that was nearly complete after 5 minutes (Fig. 2B). The extent of dephosphorylation following rapamycin treatment was greater than that seen following removal of amino acids and was close to the background level. Basal S6K phosphorylation started at a higher level, but rapamycin-induced dephosphorylation was also rapid in cells treated with PP2A catalytic subunit dsRNA. The level of S6K phosphorylation was significantly higher than control cells at both the 2 and 5 minute time points. The extent of dephosphorylation following rapamycin treatment was the same in control and PP2A-depleted cells.

Knockdown of the PP2A catalytic subunit causes apoptosis in *Drosophila* S2 cells [30,31]. In order to determine whether the induction of apoptosis contributed to effects of catalytic subunit knockdown on dS6K phosphorylation, the experiments were also carried out in the presence of the caspase inhibitor Z-VAD-fmk. Treating S2 cells with Z-VAD-fmk inhibits induction of the major hallmarks of apoptosis in S2 cells caused by depletion of PP2A subunits [32]. Treating S2 cells with Z-VAD-fmk blocked induction of apoptosis, but had no effect on the increase in basal dS6K phosphorylation or the decrease in dephosphorylation caused by knockdown of the PP2A catalytic subunit (not shown). This result indicates that the effects of catalytic subunit knockdown on dS6K phosphorylation are independent of the apoptotic response occurring downstream of caspase activation.

Knockdown of PP4 or PP6 Does Not Enhance Phosphorylation of *Drosophila* S6K

The potential involvement of *Drosophila* PP4 and PP6 in regulating S6K phosphorylation was also tested using RNA interference. The effectiveness of the dsRNA treatments in knocking down the PP4 (Fig. 3B, inset) and PP6 (Fig. 3D, inset) catalytic subunits were determined by RT-PCR since antibodies recognizing the *Drosophila* proteins were not available. The mRNA for these proteins was not detectable after 72 hours of dsRNA treatment. In contrast to the effects of PP2A knockdown, treatment of S2 cells with dsRNA for the dPP4 catalytic subunit resulted in a 20% decrease in the basal level of S6K phosphorylation relative to non-treated cells or cells treated with the control dsRNA (Fig. 3A and B). A decreased level of S6K phosphorylation was also seen at the initial time points following removal of amino acids. However, by 20 minutes when the plateau level of S6K phosphorylation had been reached, the levels of S6K phosphorylation in cells depleted of dPP4 were similar to those in cells treated with control dsRNA. Although phosphorylation started at a lower basal level, the rate and extent of S6K dephosphorylation following rapamycin treatment was similar in cells treated with either PP4 or control dsRNA. These observations suggest that *Drosophila* PP4 is not directly involved in dephosphorylation of dS6K at Thr398 since loss of a dS6K phosphatase would be predicted to enhance phosphorylation. The decreased level of basal dS6K phosphorylation seen in PP4 depleted cells raises the possibility that PP4 acts upstream of dS6K in the dTOR signaling pathway.

RNA interference was also used to assess the contribution of *Drosophila* PP6 toward dephosphorylation of dS6K. Depletion of the PP6 catalytic subunit had no effect on either the basal level of phosphorylation or the dephosphorylation of S6K following removal of amino acids or rapamycin treatment (Fig. 3C and D). This result indicates that PP6 is unlikely to play a significant role in dephosphorylation of S6K in *Drosophila* S2 cells.

Effects of Depleting PP2A Regulatory Subunits on dS6K Phosphorylation

The most common forms of PP2A are heterotrimeric holoenzymes that are targeted to individual substrates by their regulatory subunits. In order to determine if one or more of the *Drosophila* PP2A regulatory subunits were involved in targeting PP2A to dS6K, each of the known *Drosophila* regulatory subunits was knocked down by RNA interference and the effects on Thr398 phosphorylation determined following removal of amino acids.

Depletion of the *Drosophila* B α /PR55 regulatory subunit (Fig. 4A, inset) did not have a statistically significant effect on basal phosphorylation or dS6K dephosphorylation caused by amino acid starvation (Fig. 4A). However, there was a trend toward lower levels of S6K phosphorylation at each of the time points compared to cells treated with control dsRNA. Treating S2 cells with B56-1 dsRNA led to a loss of the B56-1 protein (Fig. 4B, inset). As reported previously [30,31] knockdown of the PP2A catalytic subunit also caused a loss of the B56-1 subunit due to instability of the free protein. Depletion of the B56-1 protein had no detectable effect on the basal level of dS6K or the dephosphorylation caused by amino acid

starvation (Fig. 4B). Treating S2 cells with B56-2 dsRNA resulted in a substantial, but incomplete, knockdown of B56-2 mRNA (Fig. 4C, inset). Depletion of the B56-2 subunit did not alter the basal level of dS6K phosphorylation. However, knockdown of B56-2 caused a significant increase in the rate and extent of dS6K dephosphorylation following removal of amino acids (Fig. 4C). Depletion of the PR72 regulatory subunit had no effect on either basal phosphorylation or dephosphorylation of dS6K following removal of amino acids (Fig. 4D).

Previous studies showed that the two *Drosophila* B56 subunits cooperate to promote cell survival [30–32]. B56-1 and -2 could also cooperate to regulate the phosphorylation of dS6K. This possibility was tested by knocking down both subunits simultaneously. The level of dS6K phosphorylation in cells depleted of both B56 subunits was lower than that in control cells. However, the decrease in basal phosphorylation and increased dS6K dephosphorylation during amino acid starvation were similar to those observed in cells depleted of B56-2 alone. This result indicates that B56-1 does not cooperate with B56-2 to regulate phosphorylation of dS6K at Thr398.

Depletion of dTap42 Does Not Alter Phosphorylation of dS6K

In order to test whether dTap42 was involved in targeting the PP2A catalytic subunit to S6K, the effects of depleting dTap42 on the phosphorylation of dS6K were determined. Treatment of S2 cells with dTap42 dsRNA led to a decrease in dTap42 mRNA to a level that was less than 10% of that in non-treated cells (Fig. 5B, inset). Depletion of dTap42 did not significantly alter the basal level of dS6K phosphorylation, nor did it change the rate or extent of dephosphorylation induced by amino acid starvation (Fig. 5A and B). Depletion of dTap42 also had no significant effect on dephosphorylation of dS6K caused by treating S2 cells with rapamycin. Although knockdown of dTap42 by itself did not affect dS6K phosphorylation, it was possible that dTap42 cooperates with the PP2A catalytic subunit to dephosphorylate dS6K. This possibility was tested by knocking down both proteins simultaneously. Since depletion of the PP2A catalytic subunit [30,31] or dTap42 can cause apoptosis in S2 cells, these experiments were conducted in the presence of the caspase inhibitor Z-VAD-fmk. Treatment with Z-VAD-fmk blocks the induction of apoptosis caused by knockdown of the PP2A catalytic subunit in S2 cells [32]. Cells depleted of both the PP2A catalytic subunit and dTap42 had a basal level of dS6K phosphorylation that was elevated relative to cells treated with control dsRNA (Fig. 5C and D). The rate and extent of S6K dephosphorylation following removal of amino acids was reduced in cells depleted of both proteins. Both the increased basal phosphorylation and decreased dephosphorylation caused by the double knockdown were similar to the effects of knocking down the catalytic subunit by itself (Fig. 2). These results further support the conclusion that dTap42 does not act to target PP2A to S6K in a manner that enhances dephosphorylation of Thr398.

DISCUSSION

Although members of the PP2A subfamily of serine/threonine phosphatases have been implicated in the TOR signaling pathway, the identity of the phosphatase that dephosphorylates the TOR phosphorylation site on S6K has not been determined. This study supports the conclusion that this site is dephosphorylated by PP2A, and not by other members of the PP2A subfamily or other classes of protein phosphatase. The ability of a PP2A-selective concentration of calyculin A to nearly completely block dephosphorylation of dS6K argues that only the PP2A subfamily is involved in dephosphorylating dS6K. The knockdown experiments revealed that PP2A is the only member of this subfamily that is likely to play a direct role in dephosphorylation of Thr398. A direct role for PP2A in dephosphorylating S6K is consistent with data showing that the catalytic subunit of PP2A can be isolated in complexes with mammalian S6K [20,21] and previous experiments showing that knockdown of PP2A in

Drosophila S2 leads to enhanced basal dS6K phosphorylation [18]. While the data are consistent with a direct role of PP2A in dephosphorylation of Thr398, the effect of PP2A dsRNA could also be due to altered phosphorylation of another component of the dTOR pathway.

While the loss of PP2A substantially increased S6K phosphorylation levels, RNAi-mediated depletion had a partial effect. The increase in basal dS6K phosphorylation was moderate compared to calyculin A treatment and the inhibition of dephosphorylation induced by removal of amino acids was not complete. The residual dephosphorylation was most likely due to incomplete knockdown of the PP2A catalytic subunit. Under basal conditions (complete medium), dTOR is active and a partial decrease in phosphatase activity would result in elevated steady-state levels of dS6K phosphorylation. Following removal of amino acids, TOR activity would be decreased leading to a new steady level of dS6K phosphorylation that would also be elevated in cells depleted of PP2A. The suggestion that dTOR remains at least partially active following removal of amino acids is supported by observations that amino acids only weakly activate S6K in *Drosophila* cells [42,43]. In contrast, when dTOR activity in the TORC1 complex is strongly inhibited by rapamycin, the residual PP2A could still lead to a complete, albeit slower, dephosphorylation of S6K. While the data presented here show that PP2A plays a dominant role in dephosphorylation of the TOR site on S6K, they cannot rule out a possible contribution from another calyculin A-sensitive phosphatase.

Depletion of dPP4 by RNAi led to a 20% reduction in basal phosphorylation of dS6K and a greater extent of dephosphorylation following amino acid starvation. While they indicate it is unlikely that dPP4 plays a direct role in dephosphorylation of dS6K, these results suggest a role for dPP4 in the dTOR pathway upstream of dS6K. The decrease in dS6K phosphorylation may contribute to the 20% decrease in cell growth previously observed in S2 cells depleted of dPP4 [31]. PP4 has been implicated in a variety of regulatory processes [24], but has not been extensively studied in *Drosophila*. Reduction in dPP4 levels results in a semi-lethal phenotype in *Drosophila* early embryos and defects in microtubule assembly [44]. A decrease in dTOR signaling might also contribute to reduced viability caused by deficiency of dPP4.

Based on the currently accepted model for regulation of PP2A [26], it was expected that the catalytic subunit or core dimer would be targeted to dS6K by a known PP2A regulatory protein. Consequently, knockdown of the targeting protein should inhibit dephosphorylation of Thr398 by preventing interaction of catalytic subunit and dS6K. The *Drosophila* genome contains homologs of each of the characterized families of PP2A regulatory subunits [30,31]. Knockdown of any of the four *Drosophila* PP2A regulatory subunits did not affect the basal level of dS6K phosphorylation of Thr398 nor decrease the rate or extent of dephosphorylation induced by amino acid starvation. These results suggested that dephosphorylation of Thr398 by PP2A is not mediated by one of these proteins. Although the knockdown of each of the regulatory subunits was highly efficient, the absence of an effect on dS6K phosphorylation could be due to incomplete knockdown. Alternatively, targeting to dS6K could be mediated by a novel PP2A subunit or another PP2A interacting protein. Mammalian cells contain two related proteins, striatin and SG2NA, that also interact with the PP2A core dimer [45]. These proteins have been linked to regulation of the cytoskeleton [46], lipid raft formation [47], and non-genomic actions of the estrogen receptor [48]. BLAST searches of the *Drosophila* genome identified a gene (*Cka*) encoding a transcript with 36% identity to human striatin and SG2NA. The *Drosophila* CKA protein regulates signaling through the dJNK protein kinase pathway [49]. Although there is no evidence that striatin, SG2NA, or CKA are involved in the TOR pathway, it is possible that CKA targets *Drosophila* PP2A to dS6K. The inability of regulatory subunit knockdown to increase S6K phosphorylation could also be due to a pool of free AC core dimer that mediates dephosphorylation of Thr398.

Depletion of the *Drosophila* B56-2 regulatory subunit caused a significant increase in the rate and extent of dS6K dephosphorylation induced by amino acid starvation. The data suggest that the dB56-2/PP2A complex may act upstream of dS6K to promote dTOR activity. The *Drosophila* B56-2 gene (*widerborst/wdb*) is essential for cell viability as complete loss of WDB function is lethal. Genetic analysis has demonstrated that WDB plays roles in regulating tissue polarity during development [50] and regulation of the *Drosophila* circadian clock [51]. While the results presented here indicate that dB56-2 is not directly involved in regulating dS6K, the decrease in phospho-Thr398 levels in S2 cells depleted of the protein may reflect an additional role for dB56-2 in the regulation of dTOR activity.

Based on the homology between the yeast, *Drosophila*, and mammalian Tap42/ α 4 proteins, another candidate for targeting PP2A to dS6K was the dTap42 protein. However, knockdown of dTap42 had no detectable effect on basal phosphorylation of Thr398 or on the rate or extent of dephosphorylation caused by amino acid starvation or rapamycin treatment. In addition, co-knockdown of dTap42 had no major effect on the enhanced dS6K phosphorylation caused by knockdown of the PP2A catalytic subunit. These results are consistent with a previous study showing knockdown of dTap42 had no effect on basal phosphorylation of dS6K [18]. This study also showed that disruption of the *dTap42* gene had no effect on dTOR-mediated cell growth. The lack of effect on Thr398 phosphorylation in cells depleted of dTap42 was inconsistent with a role of this protein in directing PP2A to S6K. It has been reported that α 4/mTap42, S6K, and PP2A interact in stimulated splenic B cells [17]. The data presented here suggest that such a complex is not present in *Drosophila* cells or that it mediates dephosphorylation of sites on dS6K other than the TOR site at Thr398. As observed previously in *Drosophila* imaginal disks [18] and for the α 4/mTap42 protein in murine thymocytes [52], loss of dTap42 resulted in apoptosis of S2 cells (not shown). These observations are all consistent with an essential role for the Tap42 homologs in cell survival. Since the PP2A catalytic subunit is also essential for cell survival [30–32], it is possible that some of the pro-survival actions of this phosphatase are mediated by Tap42.

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Figure 1.

Dephosphorylation of Thr398 in dS6K is blocked by selective inhibition of the type 2A subfamily of protein phosphatases. *Drosophila* S2 cells were treated for 15 min with vehicle alone (DMSO) or with 50 nM calyculin A (Calyculin A) prior to removal of amino acids (–AA) or the addition of 20 nM rapamycin (+Rap) for the indicated times. Identical sets of control cells (C) were maintained in normal medium (with amino acids and without rapamycin) for 60 minutes in the absence of calyculin A. Cells lysates were immunoblotted with a phospho-specific antibody that recognizes phosphorylated Thr398 (P-S6K) or an antibody that recognizes total S6K (S6K).

Figure 2.

Depletion of the PP2A catalytic subunit enhances phosphorylation of dS6K. (A) S2 cells were treated with either EGFP control dsRNA (EGFP dsRNA) or PP2A catalytic subunit dsRNA (C Sub dsRNA). The cells were then either starved for amino acids (–AA) or treated with 20 nM rapamycin (+Rap) for the indicated times. Cell lysates were immunoblotted with Thr398 phospho-specific antibody (P-S6K) or total S6K antibody (S6K). Control cells (C) that were not treated with dsRNA and maintained in normal medium for 60 min were included in each experiment. (B) The relative S6K phosphorylation in cells treated with control dsRNA (open symbols) or PP2A dsRNA (solid symbols) was quantitated following removal of amino acids (circles) or rapamycin treatment (squares) as described in Methods. The data are plotted as the

mean of 4 separate experiments $\pm$ standard deviation. Points marked with an asterisk indicate values that were significantly different ($p < 0.05$) between catalytic subunit dsRNA and EGFP dsRNA-treated cells. The inset shows the level of catalytic subunit knockdown as determined by immunoblotting lysates from cells treated with EGFP control or C subunit dsRNA with antibodies against the PP2A catalytic subunit (anti-C Sub) or actin (anti-actin) as a loading control.

Figure 3.

Effects of depleting PP4 and PP6 on phosphorylation of dS6K. (A) S2 cells were treated with EGFP, PP4 (panels A and B) or *Drosophila* PP6 (panels C and D) dsRNA and analyzed for S6K phosphorylation following amino acid starvation (–AA) or rapamycin treatment (+Rap) as described in Fig. 2. Phosphorylation of dS6K was quantitated in cells treated with control (open symbols) or phosphatase dsRNA (solid symbols) following removal of amino acids (circles) or rapamycin treatment (squares) for PP4 (panel B) and PP6 (panel D) depleted cells as described in Fig. 2. Points marked with an asterisk were significantly different ($p < 0.05$) between PP4 dsRNA and EGFP dsRNA-treated cells. The insets in panels B and D show the level of PP4 or PP6 mRNA knockdown in cells treated with EGFP (EGFP) or phosphatase

(PP4 and PP6) dsRNA as determined by RT-PCR. The integrity of the RNA was verified by amplification of the same samples with primers specific for the *Stubarista* gene.

Figure 4.

Effects of depleting PP2A regulatory subunits on S6K phosphorylation. S2 cells were treated with dsRNAs corresponding to the B α (A), B56-1 (B), B56-2 (C), PR72 (D), or both the B56-1 and B56-2 regulatory subunits of *Drosophila* PP2A (E). S6K phosphorylation was quantitated and plotted as described in Fig. 2. Insets show the level of depletion of regulatory subunit mRNA (A, C, and D) determined by RT-PCR as described in Fig. 3 or depletion of B56-1 protein (B) determined by immunoblotting. The arrow on the inset in panel B shows the migration of the *Drosophila* B56-1 protein and the loss of B56-1 that occurs following knockdown of the PP2A catalytic subunit.

Figure 5.

Effects of depleting *Drosophila* Tap42 on dS6K phosphorylation. S2 cells were treated with control EGFP dsRNA, dsRNA corresponding to dTap42 (A, B), or both dTap42 and the PP2A catalytic subunit (C, D). Cells were then starved for amino acids or treated with rapamycin and analyzed for S6K phosphorylation by immunoblotting as described in Fig. 2 (A and C).

Quantitative analysis of S6K phosphorylation is shown in panels B and D. Points marked with an asterisk in panel D indicate values that were significantly different ($p < 0.05$) between cells treated with EGFP dsRNA and cells treated with both dTap42 and PP2A catalytic subunit dsRNA. The inset in panel B shows the level of dTap42 knockdown as determined by

quantitative RT-PCR and is expressed relative to control cells that were not treated with dsRNA.

TABLE 1.*Drosophila* genes and primers used for PCR and preparation of dsRNA.

Protein	Gene Symbol	Forward Primer	Reverse Primer
dPP2A-C	<i>mts</i>	TCTTGATCAATGGATTGAGC	CTGTCATCGGGATCGGACCA
dPP2A-B α	<i>tws</i>	ACATCATATCCTGCGTGGA	ACAGATTGATCCGCAGGTCTG
dPP2A-56-1	<i>Pp2A-B'</i>	GAGGAGAGCACATCCAAAGA	GGAGAAGTCGAACAGTGTGC
dPP2A-B56-2	<i>wdb</i>	TCATCAGCAGCAGTCGAATA	CAACATCTTTTCGCTTAAAC
dPP2A-PR72	<i>CG4733</i>	TAGAAAGTGCTCGTAGCTAAA	CCCAGGAGCAGAATGTGCTG
dPP4	<i>Pp4-19C</i>	CAGAAACCCGGTTGGCAAAA	CGATGCTCGCGAAAAGGCTG
dPP6	<i>PpV</i>	ACTGCTGCAAGGTCTTCGAT	CAGGAAGTAGGGCGTTGTGT
dTap42	<i>CG31852</i>	AGTTGATCGACGAGGTGTCC	GGTGGACGATGCAGATT
Stubarista	<i>sta</i>	ATGTCGGGAGGCTTAGATAT	GATCTGGTTGGTGAAGGCAC