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# Death Associated Protein Kinase Phosphorylates Mammalian Ribosomal Protein S6 and Reduces Protein Synthesis†

**Andrew M. Schumacher**[‡,§], **Anastasia V. Velentza**[‡,§], **D. Martin Watterson**[‡], and **John Dresios**[¶,*]

[‡]Center for Drug Discovery and Chemical Biology, Northwestern University, 303 E. Chicago Avenue, W-896, Chicago, IL 60611

[¶]The Scripps Research Institute, Department of Neurobiology, 10550 N. Torrey Pines Road, La Jolla, CA 92037

## Abstract

Death associated protein kinase (DAPK) is a pro-apoptotic, calcium/calmodulin regulated protein kinase that is a drug discovery target for neurodegenerative disorders. Despite the potential profound physiological role of DAPK in neuronal function and pathophysiology, the endogenous substrate(s) of this kinase and the mechanisms via which DAPK elicits its biological action remain largely unknown. We report here that the mammalian 40S ribosomal protein S6 is a DAPK substrate. Results from immunoprecipitation experiments are consistent with endogenous DAPK being associated with endogenous S6 in rat brain. When S6 is a component of the 40S ribosomal subunit complex, DAPK selectively phosphorylates it at serine 235, one of the five sites in S6 that are phosphorylated by the S6 kinase family of proteins. The amino acid sequence flanking serine 235 matches the established pattern for DAPK peptide and protein substrates. Kinetic analyses using purified 40S subunits revealed a $K_m$ value of 9 μM, consistent with S6 being a potential physiological substrate of DAPK. This enzyme-substrate relationship has functional significance. DAPK suppresses translation in rabbit reticulocyte lysate and treatment of neuroblastoma cells with a stimulator of DAPK reduces protein synthesis. In both cases, suppression of translation correlates with increased phosphorylation of S6 at serine 235. These results demonstrate that DAPK is an S6 kinase and provide evidence for a novel role of DAPK in regulation of translation.

### Keywords

Apoptosis; synaptic plasticity; translational control; protein synthesis; DAPK; ribosomal protein S6; S6 kinase

Death associated protein kinase (DAPK) is a calcium and calmodulin (CaM)-regulated serine/threonine protein kinase implicated in the pathophysiology characteristic of diverse

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*To whom correspondence should be addressed: The Scripps Research Institute, Department of Neurobiology, 10550 N. Torrey Pines Road, La Jolla, CA 92037, Tel: 858-784-2636; Fax: 858-784-2646; jdresios@scripps.edu.

§Current address: Genomics Institute of the Novartis Research Foundation (GNF), San Diego, CA 92121

neurodegenerative disorders (1-3). Therapeutic intervention with bioavailable DAPK inhibitors in clinically relevant time windows is neuroprotective in animal models, validating DAPK as a potential drug discovery target for acute and chronic brain injuries (2,4,5). DAPK is highly expressed in the hippocampus, a part of the brain that is essential for memory formation. Treatment of animals with bioavailable DAPK inhibitors results in a selective upregulation in the hippocampus of the synaptosomal scaffold protein PSD-95 (4), a marker for synaptic integrity that participates in the control of synaptic transmission and plasticity (6). Consistent with a role of DAPK in synaptic plasticity, experiments in mice showed that deletion of 74 amino acids from the catalytic domain of this kinase causes superior spatial learning compared to wild-type animals (7). These findings suggest a role for DAPK in synaptic integrity and function, but a potential DAPK substrate that is involved in such regulation has not been reported. CaM regulated protein kinase kinase (CaMKK) is required for maintenance of neuronal survival and is an endogenous DAPK substrate in brain, being down regulated by DAPK site-specific phosphorylation (8). However, there is no established linkage between CaMKK and synaptic integrity.

Long-lasting synaptic plasticity has a requirement for new protein synthesis (9,10). Protein synthesis is commonly regulated via phosphorylation of translation factors and ribosomal components (11-13). One of these components is 40S ribosomal protein S6, the major phosphoprotein in mammalian ribosomes (14), phosphorylation of which is associated with translational regulation and cell function (15-19). Interestingly, biochemical analyses demonstrated the co-presence of DAPK and S6 in synaptosomes (20), although an enzyme-substrate linkage between these two proteins has not been established.

The diverse observations that link DAPK to synaptic integrity, S6 phosphorylation to the regulation of protein synthesis and cell function, and the co-localization of DAPK and S6 in synaptosomal preparations raises the logical possibility of DAPK and S6 being potentially linked by an enzyme-substrate relationship that might have functional significance in the regulation of neuronal protein synthesis in either a physiological or pathophysiological role. We report here our initial studies that seek to fill the gap in knowledge about a potential link between DAPK and eukaryotic translation. We started with a bioinformatics based approach (21) used previously to find potential physiological substrates for DAPK in brain (8). We found that S6 has a phosphorylation site sequence that matches the pattern preferred by DAPK and is an effective substrate for DAPK based on enzyme kinetic analysis. Further, we show that DAPK phosphorylation of this site correlates with effects on translational activity. The results reveal DAPK to be a novel S6 kinase (S6K), and suggest a possible mechanism through which DAPK might affect synaptosomal protein production and elicit biological function.

## MATERIALS AND METHODS

### Preparation of DAPK and Rat Liver 40S Ribosomal Subunits

A constitutively active DAPK protein (residues 1-285) containing the kinase domain was produced in *Escherichia coli* and purified as previously described (21). Small ribosomal subunits were purified from rat liver according to Thomas et al. (22) with slight modifications. Rat liver was homogenized by douncing in homogenization buffer (20 mM

Tris (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$, 5 mg/mL heparin, 1 mM DTT) supplemented with 0.2 M sucrose and centrifuged twice for 15 min at 10,000xg at 4 °C. The soluble fraction was mixed with 1/10 volume 13% sodium deoxycholate, incubated for 15 min at 4 °C, layered over a 1/3-volume cushion of homogenization buffer containing 0.7 M sucrose on top of a 1/3 volume cushion of the same buffer containing 1.6 M sucrose. The samples were centrifuged at 55,000 rpm for 16 h at 4 °C in a Beckman Ti60 rotor. Ribosome pellets were rinsed with homogenization buffer and resuspended in dissociation buffer (20 mM Tris (pH 7.4), 500 mM KCl, 3 mM $MgCl_2$, 1 mM DTT). GTP (2 mM) and puromycin (2 mM) were added followed by incubation for 30 min at 37 °C to induce premature release of the nascent polypeptide chains from translating ribosomes and dissociation of 80S ribosomes into biologically active subunits (23, 24). The solution was centrifuged twice for 15 min at 10,000 rpm in SS34 rotor, and the supernatant was loaded on a 10-35% linear sucrose gradient in dissociation buffer and centrifuged in a Beckman SW28 rotor at 27,000 rpm for 6.5 h. A complete dissociation of 80S monosomes to 40S and 60S ribosomal subunits was obtained. The fractions corresponding to 40S subunits were pooled, diluted with homogenization buffer, and pelleted by centrifugation in a Beckman SW28 rotor at 27,000 rpm for 20 h. The 40S ribosomal subunit pellets were resuspended in homogenization buffer without heparin and stored at –80°C.

### Immunoprecipitation and Western Blotting

Soluble extracts from Sprague-Dawley rat brain (5 g) were prepared in RIPA buffer (20 mM Tris (pH7.5), 1 mM EDTA, 150 mM NaCl, 0.5% NP-40, 0.1 mM NaVO3, 1 mg/L leupeptin, 1 mg/L pepstatin, 40 μM TLCK) in a Dounce homogenizer, and centrifuged (3x15 min, 15,000xg, 4 °C) to remove insoluble components. The homogenate supernatant (100 μg) was incubated with mouse-anti-DAPK $IgG_1$ (Sigma Aldrich) for 2 h at 4 °C, and then incubated with 25 μL protein A/G agarose (Santa Cruz Biotechnology) for 1 h at 4 °C. Control immunoprecipitations were performed in parallel without DAPK antibody. Each immunoprecipitation was centrifuged for 4 min at 2000xg. Pellets were washed twice with RIPA buffer and once with 20 mM Tris (pH 7.5), 1 mM EDTA. Bound protein was eluted by boiling in SDS-PAGE sample buffer.

For Western blotting, samples prepared in SDS-PAGE sample buffer were resolved by SDS-PAGE and analyzed by Western blot analysis essentially as previously described (25), using polyclonal antibodies against phospho-S6(Ser235) (Upstate Biotechnology); phospho-S6(Ser235/Ser236), phospho-S6(Ser240/Ser244), S6 (Cell Signaling Technologies); and DAPK (Santa Cruz Biotechnology). All antibodies were used at 1:1000 dilutions.

### In vitro Kinase Assays

Activity assays were carried out essentially as described previously using the DAPK catalytic domain protein (21). Briefly, 40S ribosomal subunits were incubated with 200 μM ATP and [$\gamma^{32}$P]ATP (2.5 μCi per reaction) in assay buffer (20 mM Hepes (pH 7.5), 1 mM DTT, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 75 mM NaCl) for 30 min at 25 °C, in the absence or presence of DAPK (0.13 μM) or p70 S6K (1 ng per μl reaction). Reactions were initiated by addition of DAPK or p70 S6K. Substrate depletion under these conditions was less than 10% allowing accurate determination of the $K_m$ values (8,21). To ensure that reaction rates

did not depend on the concentration of ATP, the latter was used in a concentration (200 μM) that is significantly higher that the $K_m$ value of ATP for DAPK (2 μM) (21). Whenever indicated, 40S ribosomal subunits were treated with Calf Intestinal Alkaline Phosphatase (0.1 unit per μl reaction) in assay buffer for 1 h at 37 °C. The phosphatase reaction was terminated by the addition of Phosphatase Inhibitor Cocktail (Sigma) prior to the addition of ATP and DAPK or p70 S6K. For kinetic experiments, reactions were spotted onto P-81 paper (Whatman), washed with 75 mM $H_3PO_4$ and 95% ethyl alcohol, and quantified by scintillation counting in EcoScint O (National Diagnostics). Data were plotted on a double-reciprocal graph and analyzed by linear regression using the Prism software package (Version 3.03, Graphpad Software Inc.). To verify results from double reciprocal plots, data were also analyzed by non-linear curve fitting to the Michaelis-Menten equation weighted by $1/V^2$ using Prism. For phosphoimaging experiments, reactions were terminated by adding SDS-PAGE sample buffer (100 mM Tris (pH7.0), 2% SDS, 10% glycerol, 5 mM dithiothreitol), resolved by SDS-PAGE, and proteins stained with coomassie blue R-250 to verify equal protein loading. Incorporation of $^{32}P$ was visualized by phosphoimaging (STORM, Amersham Biosciences). Stoichiometry experiments were performed using 1.0 μM 40S ribosomal subunits incubated with 0.5 μM DAPK. For phosphorylation site mapping experiments, reactions were performed as described for phosphoimaging, except only cold ATP was used, and probed by Western blotting as described above.

### In vitro Translation Measurements

Nuclease-treated rabbit reticulocyte lysate (Promega) was phosphorylated by DAPK *in vitro* under the conditions described above. Briefly, reticulocyte lysate was incubated with 200 μM ATP in assay buffer (20 mM Hepes (pH 7.5), 1 mM DTT, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 75 mM NaCl) for 30 min at 25 °C, either with or without the DAPK catalytic domain protein (0.8 μM). Aliquots of each reaction were prepared in SDS-PAGE sample buffer for Western analysis of S6 phosphorylation. The remaining reaction mixtures were used for *in vitro* translation.

*In vitro* translation reactions of reticulocyte lysates were performed essentially as described by the manufacturer (Promega). Reticulocyte lysates, with or without prior treatment with DAPK, were incubated with 8 μCi [$^{35}S$]-Methionine (Amersham Biosciences), non-radioactive amino acids (without Methionine, Promega), RNasin ribonuclease inhibitor (Promega), and 0.1 μg human liver polyA mRNA (Clonetech) for 90 min at 30 °C. Control reactions were performed without mRNA to determine background level of incorporation. Reactions were terminated by mixing 5 μL of each reaction with 95 μL 1 M NaOH/2% $H_2O_2$ and incubating for 10 min at 37 °C. Translation products were precipitated by adding 900 μL 25% trichloroacetic acid (TCA, Sigma Aldrich)/2% casamino acids (Difco) and incubating for 30 min on ice. Total counts present in each reaction were obtained by spotting 10 μL of the precipitation onto a glass fiber filter (Whatman GF/A) and scintillation counting in EcoScint O (National Diagnostics). To determine [$^{35}S$]-Methionine incorporation into translation products, 500 μL of the precipitation mixture was vacuum filtered through a GF/A filter, rinsed three times with ice-cold 5% TCA, once with acetone, and scintillation counted in EcoScint O. The percentage of radioactivity incorporation in each reaction was determined by dividing the counts in the washed products by the total

counts in the reaction. Background incorporation levels determined from reactions lacking mRNA were subtracted from incorporation values determined in the presence of mRNA. Statistical analysis was performed to compare reactions without DAPK to reactions with DAPK using Student's t-test. Significance was taken as $P<0.05$.

Fractionation of rabbit reticulocyte lysates was performed by centrifugation at 100,000xg for 3 h to obtain a pellet, containing ribosomes and a supernatant (S100), containing the bulk of protein synthesis factors. Ribosomes were washed with 0.5 M KCl to remove associated factors, and centrifuged at 100,000xg for 4 h to obtain a pellet (high-salt washed ribosomes) and a ribosomal salt wash (RSW) supernatant. Ribosomes were resuspended in homogenization buffer without heparin and treated with DAPK as described above. DAPK was subsequently dissociated from ribosomes following addition of 0.5 M KCl and centrifugation at 100,000xg for 4 h. Ribosomal pellets were resuspended in homogenization buffer without heparin and employed in reconstituted translation reactions using luciferase control mRNAs (Promega) upon supplementation with S100 and RSW fractions.

### Protein synthesis measurements in cell cultures

Protein synthesis was measured essentially as described previously (26). Neuroblastoma SH-SY5Y cells were plated in 24-well microplates ($2x10^4$ cells per well) in αMEM (Invitrogen) containing 5% fetal bovine serum and 1% antibiotics (Invitrogen). Two days later, media was replaced with serum-free media lacking Cysteine/Methionine (Cellect, ICN Biomedicals) and incubated for 20 min. Cells were then incubated for 1 h in the same media but containing 100 μCi/mL Tran$^{35}$S-label (ICN Biomedicals) in the presence or absence of 70 μM $C_2$-ceramide. Media was removed, cells were washed 5 times with ice-cold phosphate-buffered saline (PBS), and incubated in 1 mL ice-cold 10% (w:v) trichloroacetic acid (TCA) on ice for 1 h. Cells were washed five times with TCA and incubated overnight at 37 °C in 0.5 mL 1M NaOH. Liquid samples were neutralized with 6 M HCl and quantified by scintillation counting.

Replicate wells used for Western blot analysis were subjected to the same growth conditions except serum-free media containing Cysteine/Methionine was used and Tran$^{35}$S-label was not added. Cells were washed with PBS, collected in SDS-PAGE sample buffer, and subjected to Western blotting for S6, phospho-S6(Ser235), and phospho-S6(Ser240/Ser244) as described above.

## RESULTS

### Mammalian ribosomal protein S6 contains a putative DAPK phosphorylation site and interacts with DAPK in rat brain

Serine 235 is one of the five sites (Ser235, Ser236, Ser240, Ser244, and Ser247) in S6 that are phosphorylated by S6Ks (27,28). This residue is surrounded by a sequence (Figure 1A) that fits the pattern previously identified as one preferred by DAPK (21). To test whether S6 and DAPK are physically associated in mammalian brain, we performed coimmunoprecipitation experiments from adult rat brain, a tissue in which this kinase is abundant. DAPK was immunoprecipitated, and the complex was analyzed for the presence

of DAPK and S6 by Western blotting. As shown in Figure 1B, S6 was detected as part of the DAPK immunoprecipitate, but not in negative control immunoprecipitates. To test for possible contamination of the immunoprecipitate with other kinases that could interact and pull-down S6, such as p70 S6K and ERK (29), Western blots against these proteins were performed; no detectable signal for either kinase was observed (data not shown). The results indicated an *in vivo* interaction of the endogenous DAPK and S6, either directly or mediated by additional factors, suggesting that this ribosomal protein might be relevant to DAPK-mediated signal transduction pathways.

## Robust Phosphorylation of Mammalian S6 at Ser235

Under physiological conditions, S6, like several other ribosomal proteins, exists in the cytoplasm either as an integral part of ribosomes or as free protein (30). Since the immunoprecipitation experiments described above could not be used to determine which form of S6 interacts with DAPK or to establish unequivocal site-specific phosphorylation by DAPK, we employed reconstitution kinase experiments using constitutively active purified DAPK and 40S ribosomal subunits. To test whether S6 was phosphorylated by DAPK and whether Ser235 was a phosphorylation site, the reactions were probed by Western blotting with all available antibodies against the phosphorylated forms of the known S6 phosphorylation sites. Phosphorylation was observed at Ser235 (Figure 2A), the expected site of phosphorylation based on the DAPK preferred phosphorylation pattern (8,21). Phosphorylation was also observed when probing with an antibody against the phosphorylated Ser235/Ser236 epitope. In contrast, phosphorylation was not observed when an antibody against the phosphorylated Ser240/Ser244 epitope, formed by two sites that are phosphorylated by other S6Ks, was used. This data shows that there is an enzyme-substrate relationship between DAPK and S6 when the latter is in the context of 40S ribosomal subunits.

Physiological substrates for serine/threonine protein kinases are generally characterized by $K_m$ values between 1 and 20 μM (31). To determine whether phosphorylation of 40S ribosomal subunits by DAPK reflects this enzyme property, the $K_m$ of the reaction was determined. In these experiments, DAPK was used to phosphorylate *in vitro* varying concentrations of 40S subunits, and the product formation was quantified as described in Materials and Methods. As shown in Figure 2B, the $K_m$ value was approximately 9 μM and the $V_{max}$ was 0.16 mol phosphate/min/mol DAPK. The $K_m$ value was confirmed by non-linear curve fitting (see Materials and Methods). Phosphorylation of S6 by DAPK activity was verified by phosphoimaging analysis of the reaction mixtures by SDS-PAGE (Figure 2B, inset). Moreover, calculation of isotope incorporation over time revealed that DAPK phosphorylates the 40S ribosomal subunit with a stoichiometry of approximately 1 mol phosphate per mol of 40S subunits, consistent with previously characterized DAPK substrate proteins (8). The single site of phosphorylation in S6 by DAPK is Ser235 as shown by Western blot analysis of the 40S subunits with antibody against the phosphorylated Ser235 epitope after incubation of the subunits with this kinase (Figure 2A). Overall, the kinetic results demonstrate properties of 40S subunit phosphorylation by DAPK that are consistent with S6 being a physiological substrate of this kinase.

## Phosphorylation of S6 by DAPK does not affect Downstream Phosphorylation of S6 by p70 S6K

Ribosomal protein S6 can be phosphorylated at five serine residues *in vivo* and *in vitro* (32). Phosphorylation of S6 by S6Ks occurs in a distinct order in which Ser236 is the first residue phosphorylated, followed by Ser235, Ser240, Ser244 and Ser247 (27,33). There appears to be a difference in the pattern of S6 phosphorylation by DAPK compared to that caused by other S6Ks, with DAPK phosphorylating S6 selectively at Ser235; interestingly, this site is not the first one to be modified in the sequence of phosphorylation events induced in S6 by other S6Ks. It should be noted that differential phosphorylation of S6 has been observed in response to different stimuli. For example, different subsets of serine residues in S6 are phosphorylated by different protein kinases after incubation of isolated rat hepatocytes with insulin and glucagon (34). Also, different sites within S6 are phosphorylated in response to cAMP- and insulin-responsive pathways (35,36).

These observations prompted us to examine whether phosphorylation of S6 by DAPK affects its phosphorylation by the p70 S6K. To this end, 40S subunits were treated with calf intestinal alkaline phosphatase to reduce the basal level of phosphorylation, followed by incubation with either DAPK, or p70 S6K or both enzymes. 40S subunits treated with DAPK exhibited phosphorylation at Ser235; as expected, no phosphorylation was observed at Ser240/Ser244 (Figure 3). Treatment of the same subunits with p70 S6K induced phosphorylation of S6 at Ser240/Ser244 (Figure 3). When 40S subunits were incubated with DAPK and p70 S6K, elevation in the phosphorylation status of Ser235 and Ser240/Ser244 was observed (Figure 3). Interestingly, under our assay conditions p70 S6K did not phosphorylate S6 at Ser235 (Figure 3). This inability of p70 S6K could not be attributed to a lack of kinase activity, since the same enzyme phosphorylated S6 at Ser240/Ser244; likewise, this result could not be attributed to inactivity of the antibodies against the phosphorylated Ser235 epitope since the same antibodies detected phosphorylation by DAPK (Figure 3). Nonetheless, our data revealed that S6 phosphorylation by DAPK at Ser235 does not affect downstream phosphorylation of S6 at Ser240/Ser244 by p70 S6K. These findings support the earlier observation that residues at positions 235 and 236 in S6 do not affect the phosphorylation of downstream serine residues in the same protein by p70 S6K (37).

## DAPK Inhibits Protein Synthesis

Phosphorylation of S6 is correlated with the regulation of protein synthesis (15-19). To determine whether DAPK affects translation, we utilized the well-characterized rabbit reticulocyte lysate *in vitro* translation system, which contains the ribosomal machinery and translation factors necessary for protein synthesis. Rabbit reticulocyte lysate was treated with DAPK and subsequently used to translate human liver mRNA. Total mRNA translation levels were determined by measuring [$^{35}$S]-Methionine incorporation into synthesized proteins. As shown in Figure 4A, incubation of the reticulocyte lysate with DAPK dramatically reduced [$^{35}$S]-Methionine incorporation. Translational efficiency was inversely related to levels of phosphorylated S6 at Ser235 by DAPK detected by Western blot analysis of the lysate (Figure 4B). In contrast, phosphorylation at the additional sites in S6 recognized by other S6Ks, Ser240 and Ser244, remained at basal levels (Figure 2B),

consistent with the results of selective 40S phosphorylation by DAPK using reconstitution of purified components (Figure 1).

To find out whether DAPK affects translation in cells, we examined protein synthesis and the S6 phosphorylation status in cultured cells treated with ceramide, a known stimulator of the activity of DAPK in neuronal cells (38,39). Ceramide increases DAPK activity within 30 min after addition to cultured neuronal cells (39). SH-SY5Y neuroblastoma cells were cultured with [$^{35}$S]-Methionine, in the presence or absence of ceramide, for 1 h and protein synthesis was quantified by measuring [$^{35}$S]-Methionine incorporation into newly synthesized proteins. As shown in Figure 4C, exposure of neuroblastoma cells to ceramide reduced protein synthesis by approximately 25%. The suppression of protein synthesis correlated with increased phosphorylation of S6 at Ser235 (Figure 4D), the site of phosphorylation by DAPK. However, increased phosphorylation of S6 at the additional sites that are phosphorylated by other S6Ks (Ser240 and Ser244) was not observed under these conditions (data not shown), suggesting that S6 phosphorylation in response to ceramide was a result of DAPK activity.

To investigate whether phosphorylation of ribosomal protein S6 by DAPK is directly involved in the inhibition of translation, we compared the activity of ribosomes containing S6 having basal levels of phosphorylation with the activity of ribosomes containing S6 phosphorylated by DAPK. To this end, rabbit reticulocyte lysate was fractionated to a ribosome-enriched fraction and a supernatant (S100) containing the bulk of protein synthesis factors (Figure 5A). Ribosomes were treated with high salt followed by centrifugation, conditions that are known to remove ribosome-associated factors (40). Purified ribosomes were phosphorylated by DAPK (Figure 5B). The protein kinase was removed from ribosomes following high salt treatment and centrifugation, and the phosphorylated ribosomes were used in translation reactions upon supplementation with S100 and RSW fractions. It was found that ribosomes phosphorylated by DAPK were less active compared to ribosomes that had not been treated with the kinase (Figure 5C) or to ribosomes that had been phosphorylated by p70 S6K (data not shown). These results indicate that phosphorylation of S6 by DAPK is directly involved in the inhibition of translation.

## DISCUSSION

The research presented here reveals DAPK as a new S6K and potential regulator of protein synthesis. The results include several key findings. First, mammalian S6 is an endogenous substrate of DAPK. Both proteins were found in immunoprecipitates from rat brain and kinetic analyses show that S6 is a DAPK substrate. Further, the amino acid sequence surrounding the site of phosphorylation in S6 by DAPK matches the pattern revealed by positional scanning peptide library screening for DAPK substrate preferences (21) and by the proteomics-based mapping of DAPK phosphorylation sites in protein substrates (8). Second, DAPK phosphorylates S6 in the context of the 40S ribosomal subunit at position Ser235, but does not phosphorylate S6 at any of the other four sites phosphorylated by previously discovered S6Ks. In addition, phosphorylation of S6 by DAPK at Ser235 does not affect downstream phosphorylation of this ribosomal protein at Ser240/Ser244 by p70 S6K. Third, DAPK reduces translation by rabbit reticulocyte lysate towards background

levels. Furthermore, exposure of cultured neuroblastoma cells to ceramide, a known stimulus for up-regulation of DAPK activity and induction of cellular apoptosis, reduces overall protein synthesis. In both cases, the reduction in translational activity is correlated with phosphorylation of S6 at Ser235, the site of phosphorylation by DAPK. In addition, *in vitro* reconstitution experiments showed that phosphorylation of S6 by DAPK directly reduces protein synthesis.

An accumulating body of evidence has recently brought about a reevaluation of the role of the ribosome, placing it in the broader cellular environment and not just viewing it as an isolated protein-synthesizing apparatus (41-43). Relevant to studies of the pro-apoptotic protein kinase DAPK, cell apoptotic or survival signals affect protein synthesis through phosphorylation of translation initiation factors and ribosomal components (44). One of these components is ribosomal protein S6. Phosphorylation of S6 was first discovered by Gressner and Wool (14) as an event occurring during liver regeneration. Increased phosphorylation of S6 was also observed in rats injected with puromycin or cycloheximide (45) as well as in response to several other stimuli (34-36). Although three decades have passed since the discovery of S6 phosphorylation, the physiological role of this event remained elusive until recently. Employing knock-in mice, Meyuhas and colleagues (19) showed that alanine substitutions in all five phosphorylatable serine residues of S6 led to an increased rate of global protein synthesis, accelerated cell division, and smaller cell size. These observations led to the suggestion that protein synthesis is down regulated by S6 phosphorylation. Our finding that DAPK phosphorylation of S6 reduces protein synthesis is congruent with this recent report. However, the mechanism through which phosphorylation of S6 by DAPK affects protein synthesis is still unclear. Interestingly, despite the general increase in protein synthesis upon S6 mutagenesis at all five phosphorylatable serine residues, down regulation of some proteins was observed, suggesting that phosphorylation of S6 may increase the affinity of 40S ribosomal subunits to particular subclasses of mRNAs, promoting their efficient translation (19). In our studies, the effect of DAPK on translation was negative as measured by methionine incorporation, but it should be noted that this is a consensus effect on total protein synthesis; future studies are needed to address possible effects on the expression of single proteins that may be upregulated or downregulated upon S6 phosphorylation by DAPK.

Although the stoichiometry of our kinetic experiments indicated that only one site in 40S subunits was phosphorylated by DAPK (Ser235 in S6), the results neither exclude the possibility that S6 and/or other ribosomal proteins may consist DAPK substrates in their ribosome-free form nor that DAPK may phosphorylate protein factors involved in translation. In support, a synthetic peptide that is related to amino acids 218-249 of human S6 (Upstate Signaling) showed efficient phosphorylation by DAPK, suggesting that DAPK can potentially utilize the ribosome-free form of S6 as a substrate (unpublished data). In addition, although phosphorylation of ribosomal protein S6 by DAPK is directly involved in translational suppression, it might not solely account for the negative effect of this kinase on protein synthesis. Indeed, the extent to which ribosome activity was decreased via S6 phosphorylation by DAPK was smaller than the degree of translational inhibition caused by

treatment of rabbit reticulocyte lysates with DAPK. These results suggest the potential existence of additional DAPK substrates with functional roles in translation.

The regulation of gene expression at the translational level allows rapid and reversible changes in protein levels in response to localized changes in physiological conditions (46). Regulated local translation mechanisms are of paramount significance for rapid activity-mediated growth of synapses and synaptic plasticity. The results presented here for DAPK regulation of translational activity and the previous report (4) of DAPK effects on the levels of hippocampal PSD-95, a protein with crucial roles in synaptic integrity and plasticity, raise the possibility that this kinase might be a regulator of synaptic function and neuronal survival through an effect on translation. Although these findings support the notion that calcium/calmodulin-regulated pathways affect the molecular mechanisms that influence synaptic plasticity (47,48), clearly, much remains to be done as a follow up to the novel finding reported here that DAPK is capable of regulating protein synthesis with a possible role in neuronal function. Regardless, the key findings reported here and previously (2,5,8) provide an emerging view of DAPK as a kinase potentially capable of selective modulation of neuronal homeostasis in rapid response to diverse stimuli, with apoptosis as one possible outcome that can be attenuated by targeted therapeutics in disease-relevant events.

## Abbreviations

The abbreviations used are:

| | |
|---|---|
| **CaM** | calmodulin |
| **CaMKK** | calmodulin regulated protein kinase kinase |
| **DAPK** | death associated protein kinase |
| **S6** | 40S ribosomal protein S6 |
| **S6K** | ribosomal protein S6 kinase |
| **ERK** | extracellular signal-regulated kinase |

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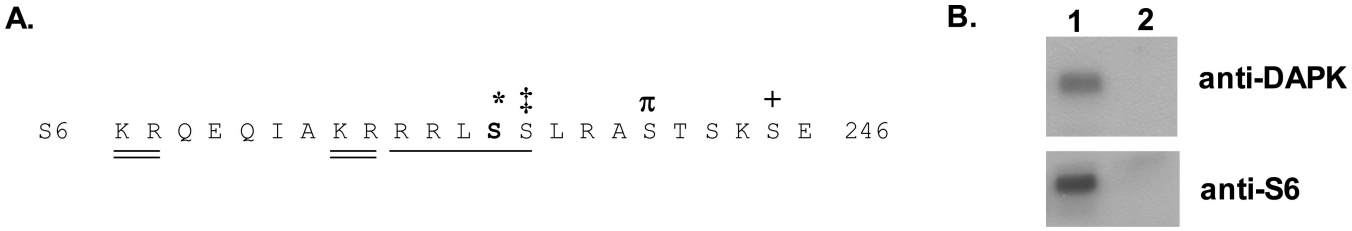


**Figure 1. Mammalian ribosomal protein S6 contains a putative DAPK phosphorylation site and interacts with DAPK in rat brain**

(A) The sequence of the human ribosomal protein S6 contains a substrate phosphorylation pattern preferred by DAPK (underscore) in which Ser235 (*) is the putative phosphorylation site. Basic residues, known to be favored in DAPK substrates upstream of the phosphorylation site, are indicated (double underscore). Other sites in ribosomal protein S6 known to be phosphorylated by S6 kinases, Ser236 (‡), Ser240 (π), and Ser244 (+), are also indicated. (B) Endogenous DAPK was immunoprecipitated from rat brain. The immunoprecipitate was analyzed by Western blotting for DAPK and ribosomal protein S6. DAPK immunoprecipitate (lane 1); control immunoprecipitate in the absence of DAPK antibody (lane 2). Data are representative of 2 independent experiments.

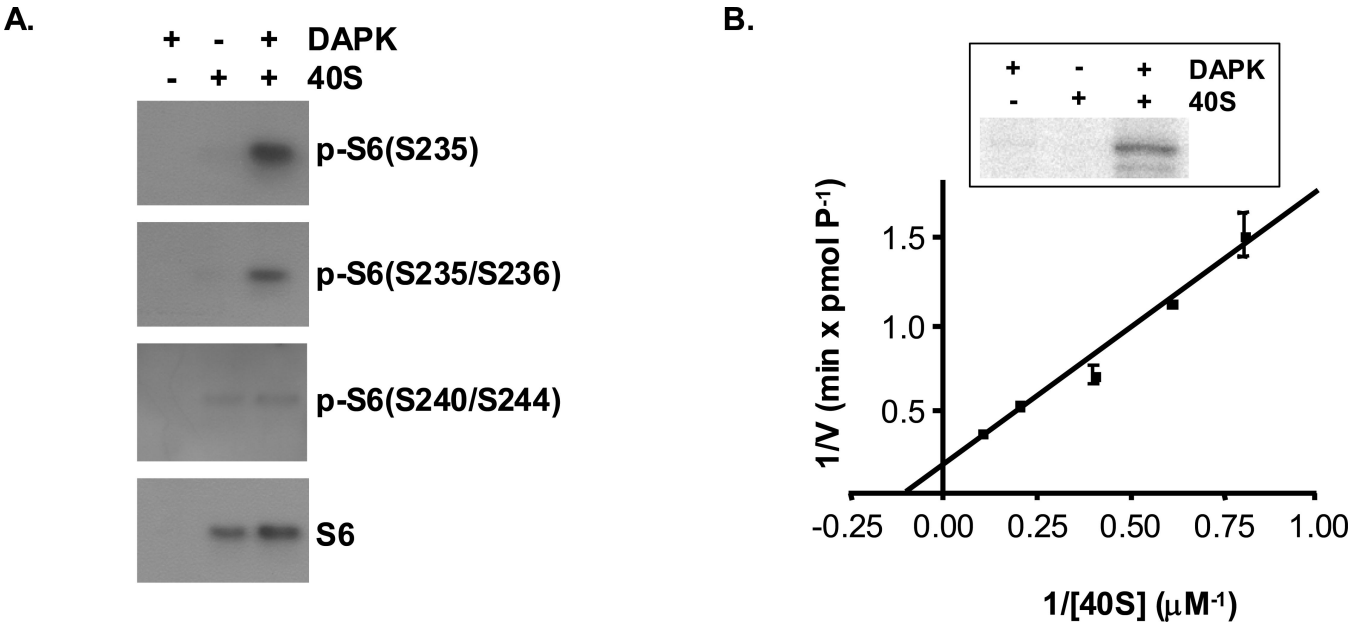


**Figure 2. Mammalian ribosomal protein S6 is a DAPK substrate**

(A) Rat liver 40S ribosomal subunits were phosphorylated *in vitro* by DAPK and analyzed by Western blotting with antibodies against phosphorylated Ser235, phosphorylated Ser235/Ser236, or phosphorylated Ser240/Ser244 epitopes. Increased phosphorylation of the Ser235 and Ser235/Ser236 epitopes, but not of the Ser240/Ser244 epitope, was observed in the presence of DAPK. Equal levels of ribosomal protein S6 were confirmed by blotting for total S6 protein. Results are representative of 2 independent experiments. (B) Constitutively active DAPK was used to phosphorylate 40S ribosomal subunits. Concentrations of 40S ranging from 1.25 μM to 10 μM were used. The $K_m$ was determined by measuring $^{32}P$-incorporation as described in Materials and Methods. Error bars represent mean values ± SEM, and are not shown if the error is smaller than the symbol. $r^2$=0.95 for the linear regression analysis. Phosphorylation of ribosomal protein S6 was verified by phosphoimaging (inset).

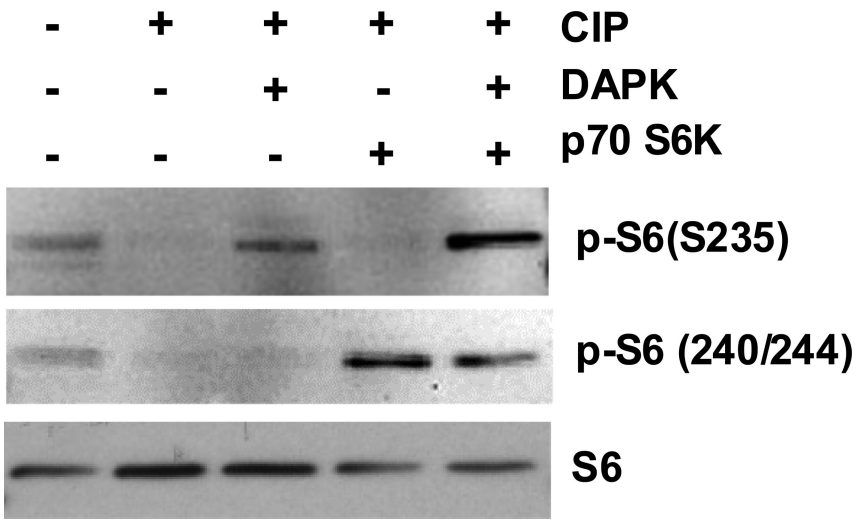


**Figure 3. Phosphorylation of ribosomal protein S6 at Ser235 by DAPK does not affect downstream phosphorylation at Ser240/Ser244 by p70 S6 Kinase**

Small ribosomal subunits from rat liver were treated with alkaline phosphatase and incubated with either DAPK, or p70 S6K, or DAPK and p70 S6K. The total amount of ribosomal protein S6, and the amount of its phosphorylated forms at Ser235 and Ser240/Ser244 was identified by Western blotting. DAPK-treated 40S subunits displayed phosphorylation at Ser235; p70 S6K-treated 40S subunits showed phosphorylation at Ser240/Ser244; and 40S subunits that had been phosphorylated by DAPK and p70 S6K showed elevated levels of phosphorylation at Ser235 as well as at Ser240/Ser244. Results are representative of 2 independent experiments.

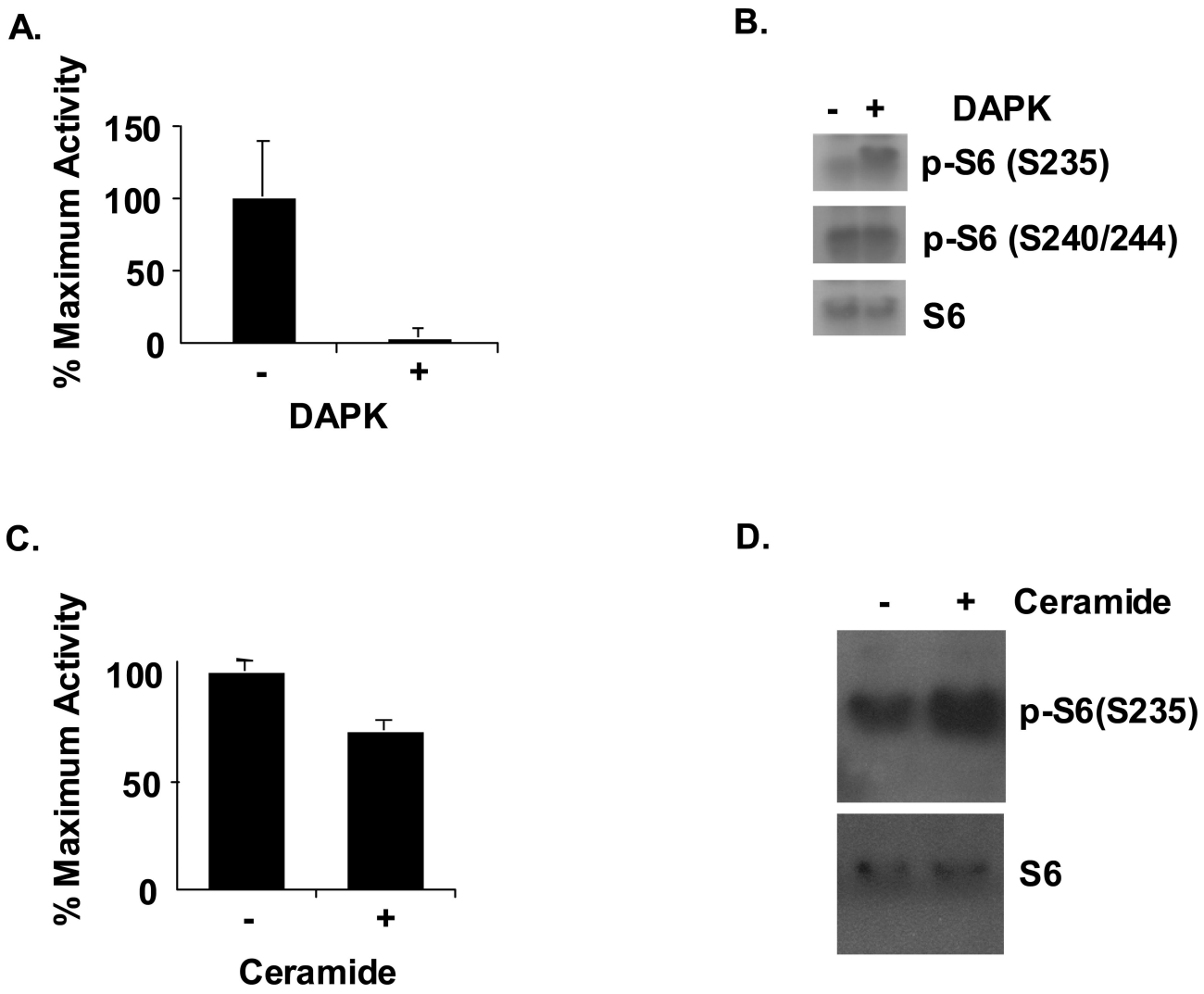


**Figure 4. DAPK inhibits translational activity**

(A) The effect of DAPK on translation *in vitro* was determined using rabbit reticulocyte lysate. DAPK was incubated with rabbit reticulocyte lysate prior to the addition of human liver mRNA and the effect of the kinase activity on translation was quantified by measuring [$^{35}$S]-Methionine incorporation into newly synthesized peptides. Data is expressed as a percentage of the maximum translation activity and is the mean ± SEM of 5 replicates from 2 independent experiments. $P < 0.05$ (*). (B) DAPK phosphorylates ribosomal protein S6 at Ser235 within the rabbit reticulocyte lysate. Samples of the reticulocyte lysate incubated in the presence or absence of DAPK were subjected to Western analysis. Equal amounts of reticulocyte lysate were present in each sample. Data shown are representative of two independent experiments. (C) The effect of ceramide, a stimulator of DAPK, on protein synthesis and ribosomal protein S6 phosphorylation were determined in SH-SY5Y neuroblastoma cell cultures. Incorporation of [$^{35}$S]-Methionine after 1 h treatment of cells with ceramide was quantified. Data is the mean ± SEM and is representative of 2 independent experiments. $P = 0.001$ (*). (D) Phosphorylation of ribosomal protein S6 at

Ser235 in response to 1 h treatment with ceramide was determined by Western blotting. Equal protein in each lane was verified by blotting for ribosomal protein S6.

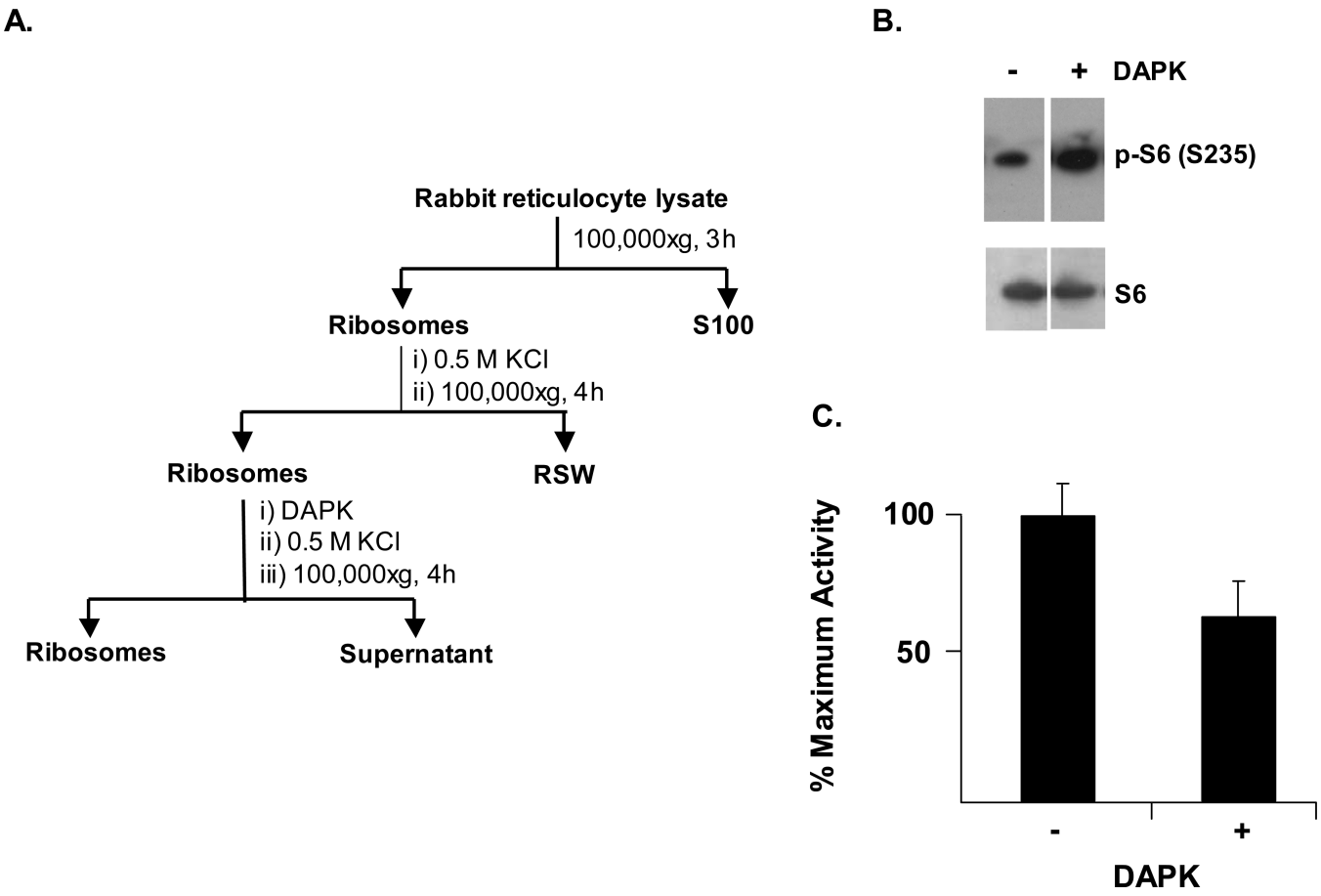


**Figure 5. Phosphorylation of S6 by DAPK is directly involved in the inhibition of translation**

(A) Schematic representation of the fractionation protocol followed to dissect the inhibitory effect of DAPK on protein synthesis (for details, see Materials and Methods). (B) Phosphorylation of ribosomal protein S6 at Ser235 following treatment of salt-washed ribosomes with DAPK was determined by Western blotting. Equal protein in each lane was verified by blotting for ribosomal protein S6. (C) Translational activities of rabbit reticulocyte reconstituted systems. Supernatant at 100,000xg (S100) and ribosomal salt wash (RSW) fractions were supplemented before the translation reaction with ribosomes treated with either no kinase (control) or DAPK. Data shown are representative of three independent experiments.