

RAS/ERK Signaling Promotes Site-specific Ribosomal Protein S6 Phosphorylation via RSK and Stimulates Cap-dependent Translation*

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Converging signals from the mammalian target of rapamycin (mTOR) and phosphoinositide 3-kinase (PI3K) pathways are well established to modulate translation initiation. Less is known regarding the molecular basis of protein synthesis regulated by other inputs, such as agonists of the Ras/extracellular signal-regulated kinase (ERK) signaling cascade. Ribosomal protein (rp) S6 is a component of the 40S ribosomal subunit that becomes phosphorylated at several serine residues upon mitogen stimulation, but the exact molecular mechanisms regulating its phosphorylation and the function of phosphorylated rpS6 is poorly understood. Here, we provide evidence that activation of the p90 ribosomal S6 kinases (RSKs) by serum, growth factors, tumor promoting phorbol esters, and oncogenic Ras is required for rpS6 phosphorylation downstream of the Ras/ERK signaling cascade. We demonstrate that while ribosomal S6 kinase 1 (S6K1) phosphorylates rpS6 at all sites, RSK exclusively phosphorylates rpS6 at Ser^{235/236} *in vitro* and *in vivo* using an mTOR-independent mechanism. Mutation of rpS6 at Ser^{235/236} reveals that phosphorylation of these sites promotes its recruitment to the 7-methylguanosine cap complex, suggesting that Ras/ERK signaling regulates assembly of the translation preinitiation complex. These data demonstrate that RSK provides an mTOR-independent pathway linking the Ras/ERK signaling cascade to the translational machinery.

In eukaryotic cells, the main rate-limiting step of translation is initiation, which is controlled by an array of proteins that respond to signaling cascades activated by extracellular signals (reviewed in Refs. 1–3). The mammalian target of rapamycin,

mTOR,⁴ is a conserved serine/threonine kinase that integrates signals from nutrients, energy sufficiency, and growth factors to regulate mammalian cell growth (reviewed in Refs. 4, 5–8). Under conditions of nutrient and energy sufficiency and insulin or mitogen stimulation, mTOR stimulates two important translational regulators, the ribosomal S6 kinases (S6K1 and S6K2) and the eukaryotic initiation factor 4E (eIF4E). eIF4E is crucial for ribosome recruitment as it binds to the 7-methylguanosine cap structure (m⁷GpppN, where N is any nucleotide) at the 5'-end of nearly all transcribed mRNAs to initiate cap-dependent translation (reviewed in Ref. 7). When mTOR is active, eIF4E nucleates the assembly of the translation preinitiation complex through recruitment of numerous initiation factors, resulting in association of the ribosomal subunits to the mRNA. S6K1 and S6K2 are serine/threonine kinases directly stimulated by mTOR which in turn, phosphorylate substrates involved in cell and body size (5, 6). S6K1 phosphorylates several substrates located in the cytoplasm and the nucleus, including the ribosomal protein (rp) S6 (reviewed in Ref. 9).

Ribosomal protein S6 is one of 33 proteins that comprise the 40 S ribosomal subunit and represents the most extensively studied substrate of S6K1 (10). Because the initial discovery that liver-derived rpS6 was phosphorylated (11), mitogenic stimulation of cells was found to correlate with phosphorylation of rpS6 on serines, which suggested that rpS6 may control mRNA translation in dividing cells (12). rpS6 phosphorylation sites have been mapped to five clustered residues that are conserved in metazoans, consisting of Ser²³⁵, Ser²³⁶, Ser²⁴⁰, Ser²⁴⁴, and Ser²⁴⁷, located at the C-terminal part of the protein (13). Two classes of protein kinases were found to phosphorylate rpS6 *in vitro*, the S6K1/2 and the p90 ribosomal S6 kinase (RSK) family of serine/threonine kinases (reviewed in Refs. 14 and 15). Subsequent studies determined that rpS6 phosphorylation was largely sensitive to the mTOR inhibitor rapamycin, indicating that S6K1/2 were the main physiological rpS6 kinases operating

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⁴ The abbreviations used are: mTOR, mammalian target of rapamycin; RSK, p90 ribosomal S6 kinase; ERK, extracellular signal-regulated kinase; S6K1, ribosomal S6 kinase 1; 4E-BP, 4E-binding proteins; rpS6, ribosomal protein S6; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; siRNA, short interfering RNA; PMA, phorbol 12-myristate 13-acetate; fmK, fluoromethylketone; GST, glutathione S-transferase; wt, wild type; EGF, epidermal growth factor; HA, hemagglutinin.

in somatic cells (16–18). The RSK family members, in contrast, are not affected by rapamycin as they are activated via the classical mitogen-activated protein kinase (MAPK) signaling pathway. The contribution of S6K1/2 in rpS6 phosphorylation was recently addressed using S6K1/S6K2 double knock-out animals, which were found to display no phosphorylation of rpS6 at Ser^{240/244}, but persistent phosphorylation at Ser^{235/236} (19). Phosphorylation of Ser^{235/236} was found to require extracellular signal-regulated kinase (ERK) signaling, suggesting that RSK or other kinases downstream of ERK, such as the mitogen- and stress-activated kinases (MSK1/2), contribute to rpS6 phosphorylation upon mitogen stimulation.

The functional importance of rpS6 in animals was underscored by conditional ablation of rpS6 in the liver (20). In these mice, hepatocytes failed to proliferate after partial hepatectomy due to a blockage in ribosome biogenesis and cell cycle progression. *In vivo* and *in vitro* studies have suggested that rpS6 phosphorylation exerts an effect on translation at the level of mRNA binding; initial chemical protection studies and cross-linking experiments localized rpS6 to the mRNA/tRNA binding site junction between the small and large ribosomal subunits (21). Consistent with this finding, highly phosphorylated ribosomes were found to bind and utilize both synthetic and natural mRNA more efficiently *in vitro* than unphosphorylated counterparts (22). More recently, the role of rpS6 phosphorylation was addressed through the generation of viable and fertile knock-in mice containing alanine substitutions of all five phosphorylatable serine residues in rpS6 (rpS6^{P-/-}) (23). These mice suffer from diminished levels of pancreatic insulin, hypoinsulinemia, and impaired glucose tolerance. Despite displaying an apparent increased rate of protein synthesis and accelerated cell division, mouse embryonic fibroblasts (MEFs) derived from these animals are significantly smaller than rpS6^{P+/+} MEFs. Moreover, the size of rpS6^{P-/-} MEFs, unlike wild-type MEFs, was not further decreased upon rapamycin treatment, implying that rpS6 is a critical mTOR effector regulating cell size (23). This is in contrast to data from S6K1 and S6K2 knock-outs, which suggest that neither S6K2 nor rpS6 phosphorylation are involved in the growth of islet or skeletal muscle cells (19, 20). Thus, it remains unclear whether rpS6 phosphorylation plays a role in cell growth. In addition, the nature of all inputs regulating rpS6 phosphorylation, and how this regulated event contributes to protein synthesis, requires further analysis.

In this study, we demonstrate that agonists of the ERK signaling pathway promote rpS6 phosphorylation using an mTOR-independent pathway that requires RSK activity. We found that activation of all four RSK isoforms stimulates cap-dependent translation, indicating that RSK provides an additional oncogene- and mitogen-regulated input linking the ERK signaling pathway to the regulation of translation initiation. Analysis of phosphorylation defective mutants of rpS6 indicates that Ras/ERK signaling promotes translation initiation by facilitating assembly of the preinitiation complex.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The plasmids encoding HA-tagged RSK1–3, Ras61L, Ras17N, MEK-DD, S6K1, kinase-inactive, and myristoylated RSK1 were previously described (24). Mouse

rpS6 was cloned into pKH3 in fusion with a triple HA tag. The bicistronic reporter plasmid pRL-5'-IRES-FL was kindly provided by Martin Kruger (Medizinische Hochschule, Hannover, Germany) and has been described (25). The human RSK4 cDNA was obtained from ATCC and subcloned into pKH3 in fusion with a triple HA tag. Glutathione S-transferase (GST)-rpS6 fusion protein was previously described (26). The point mutants used in this study were generated using the QuikChange methodology (Stratagene, La Jolla, CA).

Cell Culture and Transfection—HEK293E and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics, and transfected using calcium-phosphate (26) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Cells were grown for 24 h following transfection and starved of serum where indicated for 16–18 h. Starved cells were pretreated with wortmannin (100 nM), U0126 (10 μ M), rapamycin (100 nM) (Biomol, Plymouth Meeting, PA) or a fluoromethylketone (fmk) inhibitor (2–5 μ M) (27), and stimulated with either fetal bovine serum (10%), insulin (50 nM), epidermal growth factor (EGF; 25 ng/ml) (Invitrogen), PMA (50 ng/ml), or anisomycin (25 ng/ml) (Biomol) before harvesting.

For the small interfering RNA (siRNA) studies, 21-nucleotide complementary RNA with symmetrical two nucleotide overhangs were obtained from Qiagen (Valencia, CA). The DNA sequences against which double-stranded RNAs for S6K1, RSK1, RSK2, and scrambled control were created were described elsewhere (28, 29). HEK293E cells were transfected using calcium-phosphate and 0.25–0.5 μ g siRNA per 35-mm dishes. Transfection efficiency was determined to be greater than 90% using a fluorescently labeled mock siRNA. Twenty-four hours following transfection, cells were serum-starved for 16–18 h and stimulated with either serum or EGF. The fmk inhibitor was synthesized as previously reported (27).

Immunoprecipitation and Immunoblotting—Cell lysates were prepared using CLB (10 mM K₃PO₄, 1 mM EDTA, 5 mM EGTA, 10 mM MgCl₂, 50 mM β -glycerophosphate, 0.5% Nonidet P-40, 0.1% Brij 35, 0.1% deoxycholic acid, 1 mM sodium orthovanadate [Na₃VO₄], 1 mM phenylmethylsulfonyl fluoride, 5 μ g/ml of leupeptin, 10 μ g/ml of pepstatin) as described previously (26). Immunoprecipitations were carried out with the indicated antibody for 2 h followed by incubation with protein A-Sepharose CL-4B beads (GE Healthcare) or protein G beads (Sigma) for 1 h. For cap binding assays, lysates were incubated with 7-methyl-GTP Sepharose (GE Healthcare) for 2 h. Immunoprecipitates were washed three times in lysis buffer and along with total cell lysates were subjected to SDS-PAGE and electroblotted onto nitrocellulose as described previously (26, 30).

Antibodies—Anti-FLAG (M2) monoclonal antibodies were purchased from Sigma. Anti-HA monoclonal antibodies were kindly provided by Margaret Chou (University of Pennsylvania). Anti-p70S6K antibody was described previously (31). All anti-RSK isoform specific antibodies were kindly provided by Zymed Laboratories Inc. All phosphospecific antibodies were from Cell Signaling Technology (Beverly, MA), with the exception of the RSK phospho-Ser³⁸⁰, which was purchased from R&D Systems (Minneapolis, MN). Both anti-ERK1/2 and anti-avian RSK1 antibodies were described previously (26). For

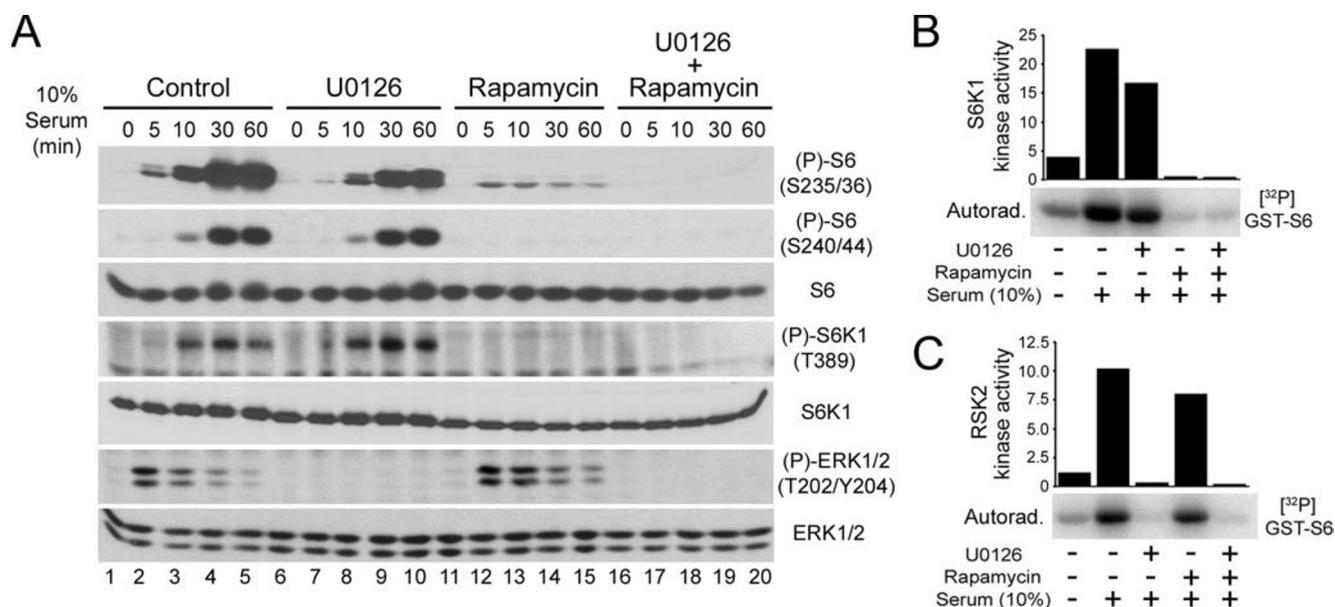


FIGURE 1. Serum stimulates rpS6 phosphorylation using mTOR- and MEK-dependent mechanisms. A, HEK293E cells were serum-starved for 18 h and pretreated with U0126 (10 μ M) and/or rapamycin (100 nM) for 30 min before stimulation with serum (10%) over a time course. Dual phosphorylation of rpS6 at Ser^{235/236} and Ser^{240/244} was monitored using phosphospecific antibodies. The cell lysates were also immunoblotted for total protein levels (rpS6, S6K1, ERK1/2), and phosphorylated S6K1 and ERK1/2. B and C, activation status of RSK2 and S6K1 was monitored in cells treated as indicated. Endogenous RSK2 and S6K1 were immunoprecipitated and assayed for their phosphotransferase activity against recombinant GST-rpS6 fusion protein *in vitro*. Relative ³²P incorporation is displayed in the histogram above the autoradiogram. These data are representative of greater than three independent experiments.

immunoblotting, anti-rabbit and anti-mouse horseradish peroxidase-conjugated antibodies were purchased from GE Healthcare and Chemicon (Temecula, CA), respectively.

Protein Kinase Assays—Beads from immunoprecipitations were washed twice in lysis buffer and twice in kinase buffer (25 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 5 mM β -glycerophosphate). Kinase assays were performed with GST-rpS6 as substrate (2 μ g per assay), and all samples were subjected to SDS-PAGE. Incorporation of cold or radioactive phosphate ³²P was determined by immunoblotting or quantified using a Bio-Rad PhosphorImager with ImageQuant software, respectively.

Bicistronic Luciferase Assays—HEK293E or HeLa cells were transiently transfected with a bicistronic luciferase reporter plasmid, pRL-5'-IRES-FL (25), which directs cap-dependent translation of the *Renilla* luciferase (RL) gene and cap-independent HCV IRES-mediated translation of the firefly (FL) gene. This construct was co-transfected along with the indicated DNA and luciferase light units were measured 48-h post-transfection using a dual luciferase assay kit (Promega, Madison, WI) and a Turner Designs TD-20/20 or BIOORBIT luminometer. Assays were performed in triplicate, and results are expressed as mean \pm S.D. from the controls.

Polysomal Fractionation—Sucrose density gradient centrifugation was employed to separate the subpolysomal from the polysomal ribosome fractions following stimulation of HEK293E cells. Five minutes before harvest, 100 μ g/ml cycloheximide was added to the culture medium. Cells were washed in ice-cold phosphate-buffered saline supplemented with 100 μ g/ml cycloheximide, and harvested in polysome lysis buffer (PLB; 50 mM HEPES (pH 7.4), 250 mM KCl, 5 mM MgCl₂, 250 mM sucrose, 1% Triton X-100, 1.3% sodium deoxycholate, 100 μ g/ml cycloheximide, and protease inhibitors). Samples were incubated on ice for 15 min and then centrifuged at 10,000 \times g

for 10 min at 4 $^{\circ}$ C. The resulting supernatant was layered on a 20–50% linear sucrose gradient (in PLB) and centrifuged in a SW40 rotor at 34,000 rpm (145,000 \times g) for 165 min at 4 $^{\circ}$ C. Following centrifugation, the A₂₆₀ was continuously monitored and recorded using a Gradient Station IP (Biocomp, Frederickton, NB) attached to a UV-MII (GE Healthcare) spectrophotometer.

RESULTS

Ras/ERK Signaling Regulates rpS6 Phosphorylation on Ser^{235/236} Using an mTOR-independent but MEK1/2-dependent Pathway—To characterize potential mTOR-independent pathways leading to rpS6 phosphorylation, HEK293E cells were stimulated with serum over a time course (Fig. 1A). Phosphorylation of rpS6 was analyzed using two phosphospecific antibodies that recognize rpS6 when dually phosphorylated on Ser^{235/236} or Ser^{240/244}. We show that, within 10 min of serum stimulation, rpS6 is robustly phosphorylated at all sites analyzed, reaching maximal phosphorylation at 30 min of stimulation (Fig. 1A, lanes 1–5). The kinetics of rpS6 phosphorylation was slightly different between the sites analyzed, with phosphorylation of Ser^{235/236} occurring with much faster kinetics than Ser^{240/244}, suggesting that phosphorylation of these sites is regulated by different mitogen-regulated signaling pathways.

When cells were pretreated with rapamycin, which completely inhibited S6K1 phosphotransferase activity (Fig. 1B) and phosphorylation at its hydrophobic motif (Thr³⁸⁹), serum did not promote rpS6 phosphorylation at Ser^{240/244}. However, Ser^{235/236} remained partly phosphorylated in cells treated with rapamycin, indicating the presence of an mTOR-independent pathway leading to rpS6 phosphorylation at these sites. To determine if the ERK signaling pathway was contributing to Ser^{235/236} phosphorylation, cells were pretreated with the MEK1/2 inhibitor U0126 before stimulation with serum.

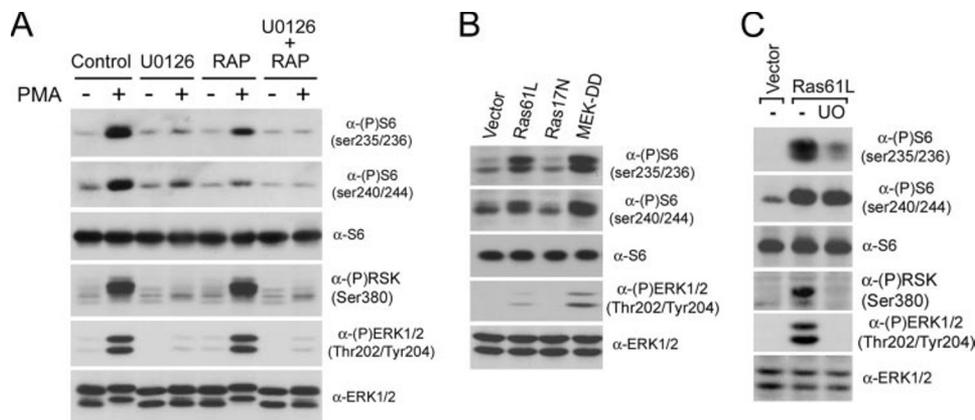


FIGURE 2. Oncogenic Ras and phorbol esters stimulate rpS6 phosphorylation using MEK-dependent mechanisms. *A*, serum-starved HEK293E cells were pretreated with U0126 (10 μ M) and/or rapamycin (100 nM) for 30 min before stimulation with PMA for 20 min. Phosphorylation of rpS6 at Ser^{235/236} and Ser^{240/244} was monitored using phosphospecific antibodies. The cell lysates were also immunoblotted for total protein levels (rpS6, ERK1/2), and phosphorylated RSK1/2 and ERK1/2. *B* and *C*, cells were transfected with control vector, oncogenic Ras (61L), dominant-negative Ras (17N), or activated MEK1 (DD), serum-starved for 16–18 h, and analyzed for rpS6 phosphorylation using phosphospecific antibodies. Total protein levels of rpS6, ERK1/2 were also determined, as well as levels of RSK1/2 and ERK1/2 phosphorylation. *C*, cells were treated with the MEK1/2 inhibitor U0126 for 30 min prior to harvesting cells for immunoblotting analysis.

To determine the role of ERK signaling in rpS6 phosphorylation, we examined the regulation of rpS6 phosphorylation in cells treated with strong inducers of the Ras/ERK signaling cascade. Stimulation of cells with the phorbol ester PMA, which stimulates Ras/ERK but not PI3K/Akt signaling in HEK293E cells, resulted in a strong induction in ERK and RSK activation, and rpS6 phosphorylation (Fig. 2*A*). Interestingly, Ser^{235/236} phosphorylation was strongly inhibited by U0126 and only partly inhibited by rapamycin, implying the more important contribution played by the ERK signaling cascade under these conditions. Next, we determined whether oncogenic Ras could promote rpS6 phosphorylation at

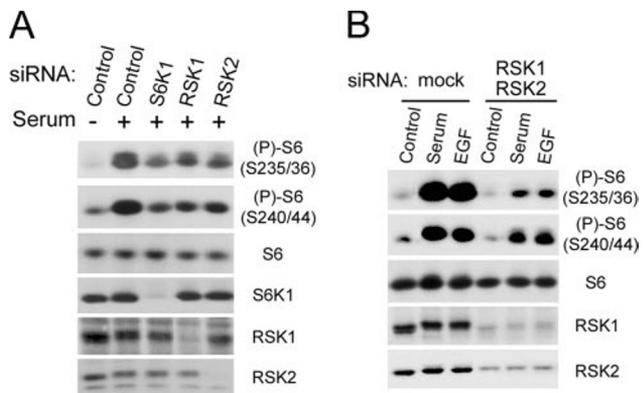


FIGURE 3. RSK1 and RSK2 are required for rpS6 phosphorylation stimulated by serum and growth factors. *A*, requirement for S6K1, RSK1, and RSK2 in HEK293E cells was determined using RNA interference. Expression of S6K1, RSK1, and RSK2 was knocked down using specific siRNAs and cell lysates were assayed for rpS6 phosphorylation at Ser^{235/236} and Ser^{240/244}. Total protein levels of rpS6, S6K1, RSK1, RSK2, and ERK1/2 were also determined, as well as levels of ERK1/2 phosphorylation. A scrambled siRNA was used as control. For sequences, see “Experimental Procedures.” *B*, HEK293E cells were transfected with scrambled control or a combination of RSK1 and RSK2 siRNAs. Cells were serum-starved for 18 h and stimulated with serum (10%) or EGF (25 ng/ml) for 15 min. Cell lysates were assayed for rpS6 phosphorylation at Ser^{235/236} and Ser^{240/244}, ERK1/2 phosphorylation, and total levels of ERK1/2, rpS6, RSK1, and RSK2.

Whereas phosphorylation of Ser^{240/244} was not significantly affected by U0126 at a concentration that efficiently blocked ERK1/2 phosphorylation (Thr²⁰²/Tyr²⁰⁴) and RSK phosphotransferase activity (Fig. 1*C*), we found that Ser^{235/236} phosphorylation was strongly reduced by the inhibitor. Interestingly, U0126 treatment resulted in a stronger inhibition of the early phase of Ser^{235/236} phosphorylation (within 10 min), suggesting a possible link between ERK signaling and the initial phase of rpS6 phosphorylation upon serum stimulation. Importantly, when rapamycin and U0126 were combined, rpS6 phosphorylation was completely inhibited, indicating that Ser^{235/236} phosphorylation occurs through mTOR- and MEK1/2-dependent mechanisms, whereas Ser^{240/244} phosphorylation is regulated predominantly via an mTOR-dependent mechanism.

Ser^{235/236}. We found that oncogenic Ras (61L), but not a dominant negative allele (17N), induced strong rpS6 phosphorylation at Ser^{235/236} (Fig. 2*B*). This stimulation was strongly inhibited by U0126 (Fig. 2*C*), indicating that ERK signaling is required for rpS6 phosphorylation under mTOR-independent conditions.

Phorbol Esters, Growth Factors, and Serum Stimulate Ribosomal Protein S6 Phosphorylation via RSK—Our data suggest that a kinase activated by Ras/ERK signaling regulates rpS6 phosphorylation at Ser^{235/236}, and the likely candidate basophilic kinases include members of the RSK and MSK families. To determine the implication of RSK in rpS6 phosphorylation, we used siRNA duplexes directed against RSK1 and RSK2, the predominantly expressed RSK isoforms in HEK293E and HeLa cells. Whereas knockdown of S6K1 reduced serum-stimulated rpS6 phosphorylation at all sites examined upon serum stimulation (Fig. 3*A*, lane 3), we found that knockdown of either RSK1 or RSK2 also reduced rpS6 phosphorylation at Ser^{235/236}, and to a lesser extent, Ser^{240/244} (Fig. 3*A*, lanes 4 and 5). This inhibition was even stronger when both RSK isoforms were simultaneously knocked down (Fig. 3*B*), indicating that RSK1 and RSK2 are normally involved in rpS6 phosphorylation upon serum and growth factor stimulation.

A fluoromethylketone (fmk) inhibitor was recently identified and characterized as an irreversible kinase inhibitor of RSK (27). We confirmed the specificity of this inhibitor and found that fmk treatment of cells specifically inhibited RSK1 and RSK2 activity by about 60%, while leaving S6K1 and MSK1/2 activity unaltered (data not shown). Similar to the data obtained using siRNAs (Fig. 3), inhibition of RSK using fmk significantly reduced rpS6 phosphorylation at Ser^{235/236}, but did not inhibit Ser^{240/244} phosphorylation (Fig. 4*A*). RSK inhibition was more effective at early time points of serum stimulation (2–10 min), which correlated with the peak of RSK and ERK1/2 activity as shown by phosphorylation at Ser³⁸⁰ and Thr²⁰²/Tyr²⁰⁴, respectively. These results implicate the RSK isoforms in the early phase of rpS6 phosphorylation at Ser^{235/236}, as previously sug-

RSK Stimulates Cap-dependent Translation

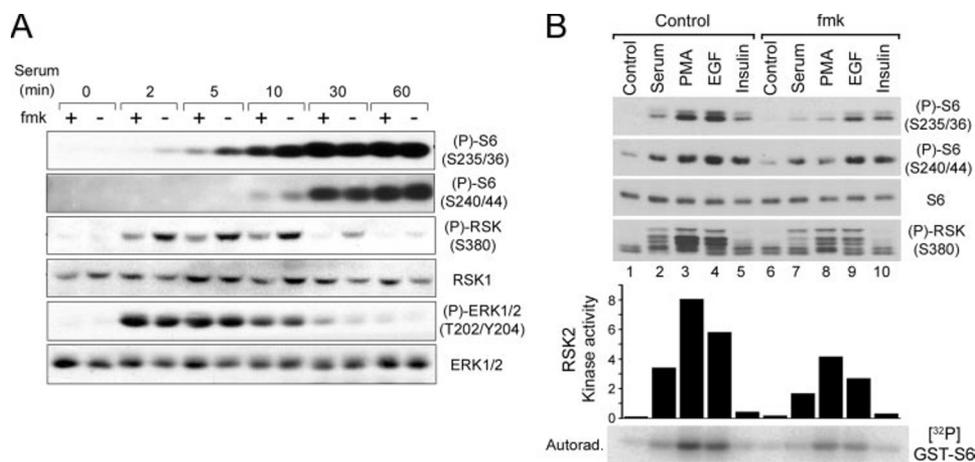


FIGURE 4. RSK activity is necessary for rpS6 phosphorylation at Ser^{235/236} in cells stimulated with serum, phorbol esters, and growth factors. *A*, requirement for RSK activity during a time course of serum stimulation was tested in HeLa cells. Serum-starved cells were treated with 2 μ M fmk for 30 min before stimulation with serum from 0–60 min. Cell lysates were assayed for rpS6 phosphorylation at Ser^{235/236} and Ser^{240/244}, RSK phosphorylation at Ser³⁸⁰, and ERK1/2 phosphorylation at Thr²⁰²/Tyr²⁰⁴. The protein levels of RSK1 and ERK1/2 were also determined by immunoblotting. *B*, requirement for RSK activity was tested in HEK293E cells by treating cells with 5 μ M fmk for 30 min before stimulating with serum (10%), PMA (50 ng/ml), EGF (25 ng/ml), or insulin (50 nM). Cell lysates were assayed for rpS6 and RSK1/2 phosphorylation, and rpS6 protein levels by immunoblotting. Immunoprecipitates of endogenous RSK2 were subjected to *in vitro* kinase activity assays using GST-rpS6 as substrate. These data are representative of greater than three independent experiments.

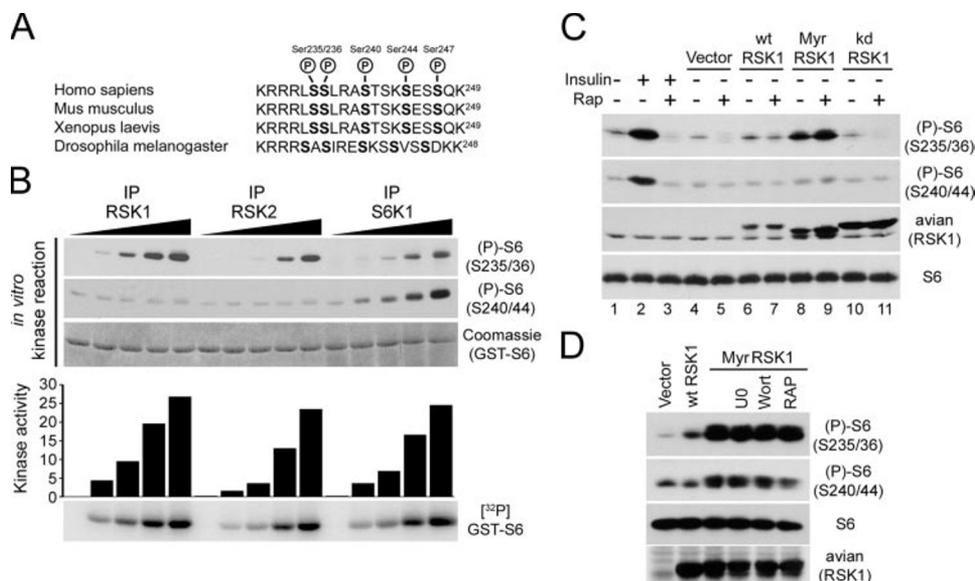


FIGURE 5. RSK1, RSK2, and S6K1 selectively phosphorylate rpS6 at different residues. *A*, alignment of the C-terminal tail of rpS6 proteins from human, mouse, frog, and fly. The identified phosphorylation sites are indicated in **boldface character** and annotated according to human numbering. *B*, the *in vitro* specificity of RSK1, RSK2, and S6K1 was tested by incubating endogenous immunoprecipitates of these kinases with bacterially derived recombinant GST-rpS6 fusion proteins. HEK293E cells were stimulated with PMA (50 ng/ml; RSK1, RSK2) or insulin (50 nM; S6K1), and increasing amounts of endogenous immunoprecipitates were assayed for kinase activity *in vitro* using phosphospecific antibodies against Ser^{235/236} and Ser^{240/244}. A portion of each sample was assayed for kinase activity using [³²P]ATP and phosphotransferase activity is displayed as an autoradiogram with corresponding histogram. The level of GST-rpS6 is shown as a Coomassie Blue-stained gel. *C*, HEK293E cells were transfected with control vector, wt RSK1, Myristoylated RSK1 (Myr), or kinase inactive RSK1 (kd), and serum-starved for 16–18 h. Quiescent cells were left untreated or treated with rapamycin (100 nM) for 30 min. A subset of cells was also stimulated with insulin (50 nM) for 15 min before harvesting. Cell lysates were assayed for rpS6 phosphorylation at Ser^{235/236} and Ser^{240/244}, and the levels of transfected RSK1 (avian isoform) and endogenous rpS6 were also determined. *D*, HEK293E cells were transfected as indicated, serum-starved for 16–18 h, and treated for 30 min with either U0126 (UO; 10 μ M), wortmannin (100 nM), or rapamycin (100 nM). Cells lysates were assayed for rpS6 phosphorylation and protein levels (*B*).

gested by the use of the MEK1/2 inhibitor U0126 (Fig. 1A). To determine whether RSK activity is required for rpS6 phosphorylation induced by other mitogenic stimuli, cells were pre-

treated with fmk before stimulation with serum, PMA, EGF, or insulin (Fig. 4B). Importantly, fmk-mediated inhibition of RSK activity significantly reduced rpS6 phosphorylation at Ser^{235/236} in response to all Ras/ERK pathway stimuli (Fig. 4B, lanes 7–9). Consistent with the requirement for Ras/ERK signaling, treatment with fmk had no effect on rpS6 phosphorylation induced by insulin, which is not a potent stimulator of RSK activity in these cells (30).

RSK1, RSK2, and S6K1 Phosphorylate rpS6 in Vitro and in Vivo with Different Specificities—The phosphorylation sites on rpS6 are located within a highly basic C-terminal region of the protein (Fig. 5A). The presence of basic residues at the –5 and –3 positions before Ser²³⁵ and Ser²³⁶ (Lys-Arg-Arg-Arg-Leu-Ser²³⁵-Ser²³⁶) locates these serines within optimal consensus phosphorylation sequences for both RSK family members and S6K1 (32). However, Ser²⁴⁰ and Ser²⁴⁴ are not preceded by such basic residues (Ser-Ser-Leu-Arg-Ala-Ser²⁴⁰-Thr-Ser-Lys-Ser²⁴⁴) indicating that they are not ideal RSK or S6K1 phosphorylation sites. To determine the specificity of RSK1, RSK2, and S6K1 toward rpS6, endogenous immunoprecipitates of these kinases were subjected to *in vitro* kinase reactions with a GST fusion protein containing the C-terminal tail of human rpS6 as substrate. Increasing amounts of kinase immunoprecipitates were tested and, as expected, RSK1, RSK2, and S6K1 were equally able to phosphorylate rpS6 on Ser^{235/236} as detected with phosphospecific antibodies (Fig. 5B). Importantly, we found that S6K1, but not RSK1 or RSK2, was capable of phosphorylating rpS6 on Ser^{240/244}. Indeed, while RSK1 and RSK2 robustly stimulated ³²P incorporation in GST-rpS6 to similar levels attained by S6K1, none of this activity was found to be targeted toward Ser^{240/244}. These results

indicate that RSK1 and RSK2 have a higher requirement for basic residues at the –5 and –3 positions, and suggest that the RSK isoforms do not directly phosphorylate Ser^{240/244} in cells.

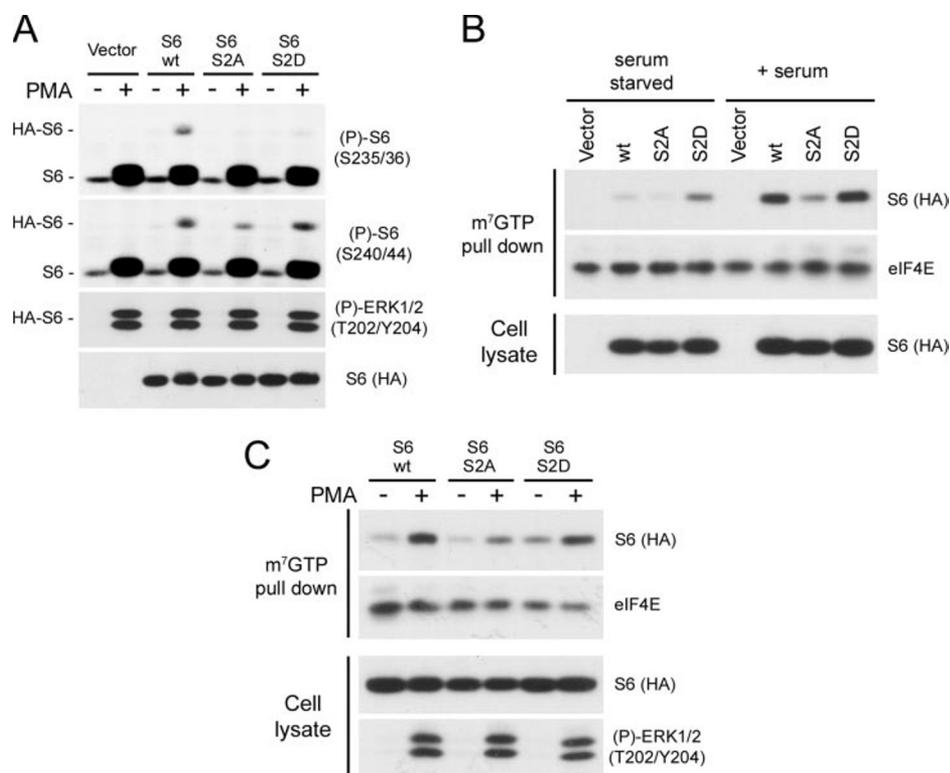


FIGURE 6. Phosphorylation of rpS6 at Ser^{235/236} promotes its association to the 7-methylguanosine cap complex. A, HEK293E cells were transfected with HA-tagged rpS6 wt, S235/36A (S2A), and S235/36D (S2D) constructs. Cells were stimulated with PMA (50 ng/ml), and total cell lysates were assayed for rpS6 phosphorylation at Ser^{235/236} and Ser^{240/244}, ERK1/2 phosphorylation, and HA-rpS6 levels. B and C, association of wt and mutant HA-tagged rpS6 proteins to the 7-methylguanosine cap complex was monitored in cells grown in the presence of serum or serum-starved for 16–18 h. Alternatively, cells were serum-starved and stimulated with PMA for 20 min. The level of associated rpS6 was determined using anti-HA immunoblotting in 7-methylguanosine precipitates. Equal levels between precipitates were monitored through analysis of eIF4E, and levels of transfected HA-rpS6 were determined in protein lysates.

To determine the kinase specificity of RSK toward rpS6 *in vivo*, cells were transiently transfected with wt, constitutively active (*Myr*), and kinase inactive (*kd*) forms of RSK1. Transfected cells were serum-starved for 18 h and assayed for basal rpS6 phosphorylation at Ser^{235/236} and Ser^{240/244} (Fig. 5C). As expected, expression of wt RSK1 (*lane 6*) or a *kd* RSK1 (*lane 10*) did not significantly stimulate rpS6 phosphorylation in serum-starved cells. However, expression of *Myr* RSK1 (*lane 8*) robustly increased rpS6 phosphorylation at Ser^{235/236}. The level of Ser^{235/236} phosphorylation was equivalent to that observed in insulin-stimulated cells, but phosphorylation at Ser^{240/244} was not significantly elevated by *Myr* RSK1 expression. We have previously found that RSK can modulate mTOR signaling through the phosphorylation and inactivation of TSC2 (24). However, we found that constitutively activated RSK1-stimulated rpS6 phosphorylation was insensitive to rapamycin treatment (Fig. 5C, *lane 9*), indicating that RSK regulates rpS6 phosphorylation using mTOR-independent mechanisms.

To determine whether constitutively activated RSK1 stimulated rpS6 phosphorylation directly, cells were pretreated with U0126, wortmannin, or rapamycin for 30 min and analyzed for rpS6 phosphorylation (Fig. 5D). We have previously shown that activation of *Myr* RSK1 is insensitive to these inhibitors (33) and as expected, rpS6 phosphorylation stimulated by *Myr* RSK1 was not affected by inhibition of mTOR, MEK1/2 or PI3K. These data clearly indicate

that the RSK isoforms can directly target the translational machinery by promoting rpS6 phosphorylation at Ser^{235/236} independently of mTOR signaling.

Phosphorylation of rpS6 Regulates Its Association to the 7-Methylguanosine Cap Complex—To determine the function of rpS6 phosphorylation at Ser^{235/236}, we generated HA-tagged rpS6 mutants with either alanine or aspartic acid substitutions at positions 235 and 236. These constructs were transfected in HEK293E cells and cell lysates were assayed for endogenous (*lower band*) and transfected (*upper band*) rpS6 phosphorylation within the same sample (Fig. 6A). As expected, while endogenous rpS6 phosphorylation was equally stimulated by PMA treatment, phosphorylation of Ser^{235/236} was completely abolished in the HA-rpS6 S235A/S236A (S2A) and S235D/S236D (S2D) mutants (Fig. 6A). To determine whether RSK-mediated phosphorylation of rpS6 promoted its recruitment to the mRNA cap complex, we tested the ability of the rpS6 mutants to bind 7-methylguanosine cap beads. We found that, both in the absence and presence of serum, binding of

the rpS6 S2A mutant was severely impaired compared with wt protein (Fig. 6B). Interestingly, binding of the rpS6 S2D mutant to cap beads was more efficient than wt protein in the absence of serum, indicating that phosphorylation of Ser^{235/236} normally promotes rpS6 binding to the 7-methylguanosine cap complex. Under conditions where wt rpS6 is phosphorylated in response to serum, both wt and the rpS6 S2D mutant were found to be recruited to cap beads with similar affinities. Next, we determined whether strong ERK signaling agonists require phosphorylation of Ser^{235/236} to recruit rpS6 to the 7-methylguanosine cap-binding complex. Whereas wt rpS6 was robustly recruited to the 7-methylguanosine cap complex in a PMA-stimulated manner, we found that mutation of Ser^{235/236} to alanine residues severely impaired binding of rpS6 to cap beads (Fig. 6C). Phosphorylation of Ser^{235/236} appeared to be important for recruitment of rpS6, as the phosphomimetic mutant of rpS6 (S2D) bound to the mRNA cap complex even in the absence of PMA stimulation. These results show that rpS6 phosphorylation at Ser^{235/236} facilitates rpS6 recruitment to the mRNA cap-binding complex, indicating that RSK-mediated phosphorylation of rpS6 may stimulate assembly of the translation initiation complex.

The RSK Isoforms Promote Cap-dependent Translation and Polysome Assembly—To determine whether the RSK isoforms are required for translation initiation, we used a dual luciferase

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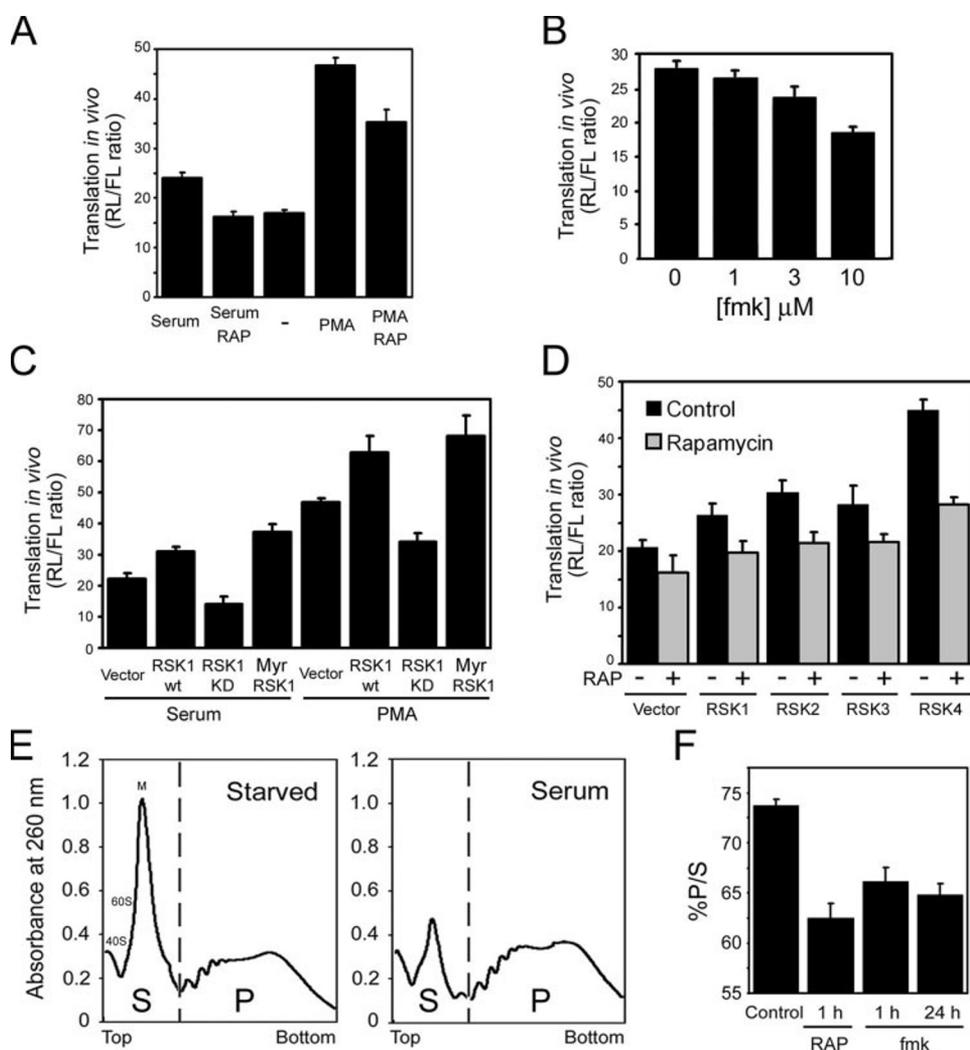


FIGURE 7. RSK stimulates cap-dependent translation and polysome formation. A bicistronic reporter plasmid that directs cap-dependent translation of the *Renilla* luciferase (RL) gene and cap-independent HCV IRES-mediated translation of the firefly (FL) gene was used to determine the role of RSK in cap-dependent translation. A and B, transfected HEK293E cells were grown in the presence of serum or serum-starved for 24 h in the presence of PMA (50 ng/ml), rapamycin (100 nM), or increasing dose of fmk. Cells were harvested and RL/FL luminescence quantified using a luminometer. C, HEK293E cells were co-transfected with the reporter vector and different RSK1 mutants. Cells were grown in serum or serum-starved for 24 h in the presence of PMA (100 ng/ml) before harvesting for luminescence. D, cells were co-transfected with the reporter vector and different RSK isoforms and grown in the presence of serum with or without rapamycin (100 nM) for 24 h. Luminescence was determined and graphed as in B. For all panels, the RL/FL luminescence ratio is expressed as a histogram \pm S.E. from three independent experiments. E, HEK293E cells were serum-starved for 24 h or grown in the presence of serum, and cellular extracts were size-fractionated by centrifugation through sucrose gradients (20–50%). The absorbance of polysomes and subpolysomal particles was continuously monitored at 260 nm, and the vertical dashed line separates the polysomal (P) and the subpolysomal fractions (S). Representative A_{260} nm traces are shown ($n = 3$). F, cells were grown in the presence of serum and incubated with rapamycin (100 nM) or fmk (5 μ M) for 24 h or 1 h before subjecting cellular extracts to sucrose gradients. The area under the curves was calculated using ImageJ (NIH), and the proportion of P/S has been referred to in the histogram as the percentage of ribosomes engaged in translation. The results are presented as a mean \pm S.E. ($n = 4$).

reporter system that monitors the ratio between cap-dependent and -independent translation initiation (25, 34). Using this system, we first confirmed that serum and PMA both stimulate cap-dependent translation (1.5- and 2.5-fold, respectively), which was significantly suppressed by rapamycin treatment because of the inhibition of eIF4E function (Fig. 7A). To determine whether RSK activity is required for cap-dependent translation, reporter construct-transfected HEK293E or HeLa cells were grown in serum-containing medium and treated for 24 h with increasing concentrations of the fmk inhibitor (Fig. 7B).

Under conditions that potently inhibit RSK activity (see Fig. 4), we found that cap-dependent translation was inhibited in a dose-dependent manner, with 10 μ M fmk leading to a 30% reduction in translation initiation (Fig. 7B). This inhibitory level was comparable to the effect of rapamycin (see Fig. 7A), suggesting that RSK activity is important for translation initiation.

To further define this observation, we measured the effect of RSK overexpression on cap-dependent translation. As shown in Fig. 7C, expression of either wt RSK1 or Myr RSK1 significantly increased cap-dependent translation over serum or PMA treatment alone. Conversely, expression of kinase-inactive RSK1 slightly inhibited cap-dependent translation, confirming that RSK1 kinase activity is necessary to stimulate cap-dependent translation. To determine whether all RSK isoforms stimulated cap-dependent translation, RSK1–4 were overexpressed in the same paradigm. Interestingly, we found that all four RSK isoforms stimulated cap-dependent translation in serum growing cells (Fig. 7D). RSK-stimulated translation initiation was found to be sensitive to rapamycin, which is consistent with the fact that mTOR activity is required for eIF4E activity and nucleation of the translation initiation complex. Together, these data demonstrate that RSK positively contributes to cap-dependent translation.

To directly examine the requirement for RSK activity in ribosomal recruitment to mRNAs, we analyzed the sedimentation of ribosomes from cells treated with the RSK inhibitor. The absorbance at 260 nm was measured throughout the resulting sucrose gradients (from top to bottom) which unveiled two regions (separated by the dashed line) composed of polysomal (P) and subpolysomal (S) fractions. As shown in Fig. 7E, serum stimulation resulted in increased P/S ratio compared with cells that were starved of serum. Interestingly, both rapamycin and fmk pretreatment for 1 h decreased the level of polysomes induced by serum stimulation (Fig. 7F), indicating that mTOR and RSK activities are important contributors to acute serum-stimulated polysome formation. Together, these data suggest that RSK provides a link between the Ras/ERK signaling cascade and the translational

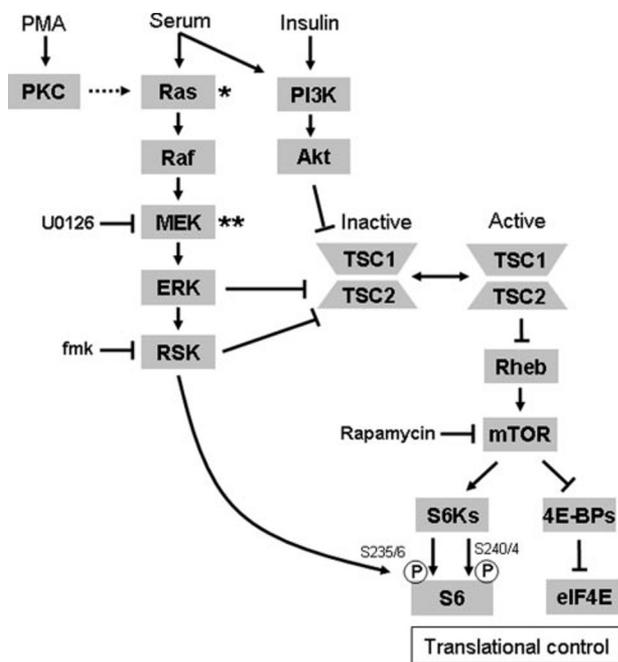


FIGURE 8. Ras/ERK signaling stimulates site-specific rpS6 phosphorylation via RSK. The growth factor stimulated signaling pathways that converge on the translational machinery to regulate cap-dependent translation are depicted in the schematic representation.

machinery by promoting recruitment of rpS6 and ribosomal subunits to the translation preinitiation complex (Fig. 8).

DISCUSSION

We have described an important link between the Ras/ERK signaling cascade and the translational machinery, which extends our current understanding of the mechanisms by which stimuli of this cascade promote protein synthesis. We found that serum, growth factors, oncogenic Ras, and phorbol esters promote rpS6 phosphorylation at Ser^{235/236} using an mTOR-independent pathway that requires RSK activity. Phosphorylation of Ser^{235/236} was found to regulate the affinity of rpS6 for the 7-methylguanosine cap complex, indicating that RSK signaling contributes to the assembly of the translation initiation complex. We demonstrated that all RSK isoforms stimulate cap-dependent translation in cells exposed to serum and phorbol esters and that RSK activity was required for efficient recruitment of ribosomes to mRNAs, indicating that RSK family members are critical effectors of translation initiation. Thus, the RSK family, mediators of Ras signaling, and the S6K family, mediators of mTOR and PI3K signaling, can collaboratively modulate protein synthesis.

RSK1 was originally identified in *Xenopus* oocytes as a serine kinase that phosphorylated ribosomal protein S6 *in vitro* (35). However, because S6K1 and S6K2 were later found to be the predominant rpS6 kinases operating in somatic cells (16, 36), RSK family members were no longer believed to be involved in rpS6 phosphorylation and translation initiation. Studies in S6K1/S6K2-null MEFs confirmed that S6K1 and S6K2 were the major rpS6 kinases, but also demonstrated that a MEK1/2-dependent kinase phosphorylated rpS6 at Ser^{235/236} (19). Consistent with these results, we found that all four RSK isoforms phosphorylated rpS6 at Ser^{235/236} but not Ser^{240/244} *in vitro* and

in vivo, consistent with the idea that S6K1/2 and the RSK isoforms converge on rpS6 to modulate mRNA translation. We have previously shown that RSK1 can phosphorylate and inactivate the TSC2 tumor suppressor, a negative regulator of mTOR signaling (24). ERK2 was also found to regulate TSC2 activity (37), indicating that Ras/ERK signaling can modulate rpS6 phosphorylation through the regulation of mTOR signaling. Here, we show that rapamycin treatment does not fully inhibit RSK-mediated rpS6 phosphorylation at Ser^{235/236}, indicating that RSK modulates rpS6 phosphorylation using TSC2/mTOR-dependent and -independent mechanisms.

A recent report by Meyuhas and co-workers (23) described a knock-in mouse (rpS6^{P-/-}) with alanine substitutions of all five phosphorylatable serines within the C terminus of rpS6. These mice suffer from diminished levels of pancreatic insulin, hypoinsulinemia, and impaired glucose tolerance, indicating that rpS6 phosphorylation is required for the synthesis or function of some critical proteins that normally repress hyperglycemia (23). Importantly, polysomal association and global protein synthesis rates were found to be altered in MEFs and livers from these mice, indicating that rpS6 phosphorylation regulates mRNA translation. The rates of protein synthesis and accumulation were found to be slightly increased in rpS6^{P-/-} MEFs, but whether this difference results from differences in protein degradation remains to be determined. In addition, rpS6^{P-/-} MEFs are significantly smaller than wild type counterparts, indicating that rpS6 phosphorylation contributes to the regulation of cell size via an unknown mechanism. Based on our findings, rpS6 phosphorylation appears to be regulated by at least two families of enzymes, the RSK and S6K. We found that the RSK isoforms specifically phosphorylate Ser^{235/236} but not Ser^{240/244}, suggesting that the sites of rpS6 phosphorylation may accomplish different molecular functions. The phenotype of the rpS6^{P-/-} mouse may represent the average of a number of altered molecular functions normally mediated by site-specific phosphorylation of rpS6.

We also show that rpS6 is recruited to the 7-methylguanosine cap binding complex more efficiently in cells with activated Ras/ERK signaling, suggesting a mechanism by which phosphorylation of rpS6 by RSK proteins promotes its recruitment to the translation preinitiation complex. These results are in complete agreement with earlier findings demonstrating that phosphorylated ribosomes interact with both synthetic and natural mRNA more efficiently than unphosphorylated counterparts (22). Our results indicate that RSK activity is important for polysome formation and presents a paradigm for the interaction between the PI3K/mTOR and Ras/ERK signaling cascades in controlling translation initiation. Because these pathways have distinct activation kinetics, their differential regulation may provide cells with a mechanism for differential regulation of common molecular targets. Cooperation between these pathways was previously shown to contribute to glioblastoma formation by stimulating the recruitment of specific mRNAs to ribosomes (38). Interestingly, the mRNAs most affected are those encoding proteins that regulate growth and metastasis, suggesting a model by which Ras/ERK and PI3K/mTOR signaling leads to cellular transformation by altering the composition of mRNAs associated with

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actively translating polysomes. Contribution of both pathways in mRNA translation has also been observed in neuronal models of synaptic plasticity (39), where inhibition of ERK1/2 and mTOR signaling was found to block neuronal activity-induced translation initiation as well as phosphorylation of rpS6.

Finally, our results raise important issues regarding the use of rpS6 phospho-Ser^{235/236} antibodies as biomarkers for activation of the mTOR/PI3K pathway when staining tissue samples from tumor biopsies as has become common practice. Although in many cases phosphorylation of these sites may be regulated predominantly by inappropriate mTOR/PI3K/S6K signaling, there are potentially many cases, for example in tumors with activated Ras or Raf, where phosphorylated Ser^{235/236} may be regulated primarily by RSK. Such data might lead to the inappropriate conclusion about the requirement of specific signaling pathways. In the future, targeted therapies may depend on the use of specific biomarkers such as phospho-ERK or phospho-RSK antibodies to reflect Ras/Raf activation and rpS6 phospho-Ser^{240/244} antibodies to reflect PI3K/mTOR activation. In addition, the use of rapamycin in treating disease may result in the development of rapamycin-resistant cancers due to the compensation by the Ras/Raf pathway. Under these conditions, inhibitors in this pathway will be therapeutically beneficial. Given additional roles of RSK in cell survival and migration (14), RSK inhibitors may prove to be beneficial in the treatment of some cancers.

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