

Transduction of Growth or Mitogenic Signals into Translational Activation of TOP mRNAs Is Fully Reliant on the Phosphatidylinositol 3-Kinase-Mediated Pathway but Requires neither S6K1 nor rpS6 Phosphorylation

Miri Stolovich,¹ Hua Tang,^{1†} Eran Hornstein,¹ Galit Levy,¹ Ruth Cohen,¹ Sun Sik Bae,² Morris J. Birnbaum,² and Oded Meyuhas^{1*}

Department of Biochemistry, The Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel,¹ and Howard Hughes Medical Institute and Cox Institute at the University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104²

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Translation of terminal oligopyrimidine tract (TOP) mRNAs, which encode multiple components of the protein synthesis machinery, is known to be controlled by mitogenic stimuli. We now show that the ability of cells to progress through the cell cycle is not a prerequisite for this mode of regulation. TOP mRNAs can be translationally activated when PC12 or embryonic stem (ES) cells are induced to grow (increase their size) by nerve growth factor and retinoic acid, respectively, while remaining mitotically arrested. However, both growth and mitogenic signals converge via the phosphatidylinositol 3-kinase (PI3-kinase)-mediated pathway and are transduced to efficiently translate TOP mRNAs. Translational activation of TOP mRNAs can be abolished by LY294002, a PI3-kinase inhibitor, or by overexpression of PTEN as well as by dominant-negative mutants of PI3-kinase or its effectors, PDK1 and protein kinase B α (PKB α). Likewise, overexpression of constitutively active PI3-kinase or PKB α can relieve the translational repression of TOP mRNAs in quiescent cells. Both mitogenic and growth signals lead to phosphorylation of ribosomal protein S6 (rpS6), which precedes the translational activation of TOP mRNAs. Nevertheless, neither rpS6 phosphorylation nor its kinase, S6K1, is essential for the translational response of these mRNAs. Thus, TOP mRNAs can be translationally activated by growth or mitogenic stimuli of ES cells, whose rpS6 is constitutively unphosphorylated due to the disruption of both alleles of S6K1. Similarly, complete inhibition of mammalian target of rapamycin (mTOR) and its effector S6K by rapamycin in various cell lines has only a mild repressive effect on the translation of TOP mRNAs. It therefore appears that translation of TOP mRNAs is primarily regulated by growth and mitogenic cues through the PI3-kinase pathway, with a minor role, if any, for the mTOR pathway.

Cell proliferation involves two processes: cell growth (increase in cell size) and cell division, which are normally intermingled, to the extent that cells must attain a minimal size to progress in the cell cycle. The dependence of DNA replication and cell division on cellular growth appears to enable accumulation of cellular resources to ensure daughter cell survival. Growth is characterized by elevated production of the translational apparatus needed to cope with the increasing demand for protein synthesis (42). Indeed, according to one estimate, most of the energy consumed during cellular growth is utilized for generating the components of the protein synthesis machinery (53).

TOP mRNAs, which encode many components of the translational apparatus [ribosomal proteins, elongation factors eEF1A and eEF2, and poly(A)-binding protein], are translationally regulated by mitogenic signals through their

5' terminal oligopyrimidine tract (5'TOP) (35). Translational repression of TOP mRNAs is apparent when proliferation of vertebrate cells is blocked by a wide variety of physiological signals (terminal differentiation, contact inhibition, and serum starvation) or by cell cycle inhibitors (aphidicolin and nocodazole). The shift of TOP mRNAs from polysomes into mRNP particles (subpolysomal fraction) under such circumstances clearly indicates that the translational repression results from a block at the translational initiation step (37).

PI3-kinase is a receptor-proximal component of a mammalian growth-regulating pathway. It has a number of downstream effectors and has been implicated in a wide variety of cellular responses (reviewed in reference 10). Stimulation of a variety of growth factor receptors leads to enhanced PI3-kinase activity and elevated levels of its products, phosphatidylinositol-3,4,5-P₃ and phosphatidylinositol-3,4-P₂. Increased levels of these lipids are also apparent by the loss of function of PTEN (phosphatase and *tenzin* homolog deleted from chromosome 10), which cleaves the D3 phosphate of phosphatidylinositol-3,4,5-P₃ and phosphatidylinositol-3,4-P₂ (reviewed in reference 34). These second-messenger lipids participate with 3-phosphoinositide-dependent kinase 1 (PDK1) in activation of

* Corresponding author. Mailing address: Department of Biochemistry, Hebrew University-Hadassah Medical School, P.O. Box 12272, Jerusalem 91120, Israel. Phone: 972 2 6758290. Fax: 972 2 6758911. E-mail: meyuhas@cc.huji.ac.il.

† Present address: Department of Biological Regulation, The Weizmann Institute of Science, Rehovot 76100, Israel.

protein kinase B (PKB), which appears to mediate downstream events controlled by PI3-kinase (reviewed in references 8 and 32).

In mammals, the three isoforms PKB α , PKB β , and PKB γ are encoded by distinct genes. PKB α -deficient mice are viable, but their growth is retarded (14). PKB β -null mice are viable but impaired in their ability to maintain normal blood glucose homeostasis (13). Likewise, *Drosophila* eye cells which are PKB (Dakt1) deficient exhibit a reduction in size (65). Overexpression of PKB α in mouse pancreatic β -cells leads to an increase in their size (64). Taken together, it appears that PKB α is a potent determinant of cell size in mammals.

One of the downstream targets of PI3-kinase is the ribosomal protein S6 (rpS6), whose phosphorylation is carried out by two closely related kinases, S6K1 and S6K2, very early following mitogenic stimuli (reviewed in reference 20). The activation of S6K relies, in addition to the PI3-kinase-mediated pathway, on the mammalian target of rapamycin (mTOR; also known as FRAP or RAFT) (reviewed in reference 35). The fact that this event precedes the translational activation of TOP mRNAs has led to a model which attributes the translational efficiency of these mRNAs to S6K activity and rpS6 phosphorylation (26, 27, 62). This model has been based on two lines of evidence. First, inhibition of S6K1 and S6K2, and consequently of rpS6 phosphorylation by the immunosuppressant rapamycin (an mTOR-specific inhibitor) led to suppression of the mitogenic activation of TOP mRNA translation in a selected set of cell lines (reviewed in reference 23). Second, overexpression of a dominant-interfering mutant of S6K1 has been shown to exert a minor inhibitory effect on the translational activation of a chimeric TOP mRNA following mitogenic stimulation (25).

It has recently been shown that in addition to their regulation by mitogenic stimulation, TOP mRNAs are translationally controlled by amino acid sufficiency (58). However, this mode of regulation requires neither S6K1 activity nor rpS6 phosphorylation (58). Moreover, inhibition of mTOR by rapamycin led to fast and complete repression of S6K1, as judged from the lack of rpS6 phosphorylation, but to only partial and delayed repression of translational activation of TOP mRNAs (58). These findings, together with the rapamycin resistance of TOP mRNAs in some cell lines (26, 28), have cast doubts on the role of the mTOR-mediated pathway in the translational control of TOP mRNAs.

Here we show for the first time that TOP mRNAs are translationally activated when cells are induced to grow, even in the absence of cell proliferation. Experiments based on multiple strategies have disclosed that transduction of both growth and mitogenic signals into translational efficiency of TOP mRNAs is absolutely dependent on the PI3-kinase-mediated pathway. In contrast, S6K activity and rpS6 phosphorylation are fully dispensable for this mode of regulation, and the inhibition of mTOR has only a minor, if any, repressive effect on the translational efficiency of TOP mRNAs.

MATERIALS AND METHODS

Cell culture and DNA transfection. PC12 cells were grown in 100-mm plates in Dulbecco's modified Eagle's medium supplemented with 8% fetal calf serum, 8% donor horse serum, 100 U of penicillin per ml, and 0.1 mg of streptomycin

per ml. Neuronal differentiation was induced with 25 ng of mouse nerve growth factor per ml (Alomone Labs). Cells were starved for serum by removal of the growth medium, washing once with 5 ml of phosphate-buffered saline and incubation with serum-free medium for the indicated times. Rapamycin (Sigma) and LY294002 (Sigma) were added at 20 nM and 50 μ M, respectively.

Akt1/PKB α ^{+/+} and Akt1/PKB α ^{-/-} mouse embryo fibroblasts (MEF) were derived as described previously (63) and grown in 100-mm plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U of penicillin per ml, and 0.1 mg of streptomycin per ml. R1 mouse embryonic stem (ES) cells and their S6K1-deficient derivative cells (p70S6K^{-/-}) were kindly provided by Naohiro Terada and grown as described previously (58).

Neuronal differentiation of S6K^{-/-} ES cells was performed as previously described (5, 6). Briefly, cells were trypsinized and transferred into bacterial plates, in which they spontaneously aggregated into embryoid bodies. Four days of incubation in the absence of retinoic acid were followed by 4 days of incubation in its presence (0.5 μ M), and then cells were transferred into tissue culture plates. The proliferation of dividing or differentiating S6K^{-/-} ES cells was quantified by the methylene blue staining protocol (41). Human embryonic kidney 293 cells were grown in 100-mm plates and transfected as described previously (22). Mitotic arrest of MEF and S6K^{-/-} ES cells was achieved by incubation in serum-free medium for 44 h. 293 cells were serum starved by first transferring cells into bacterial plates and then incubating in serum-free medium for 26 h.

Mitotic stimulation of these cells was carried out by serum refeeding after they had been transferred back into cell culture plates. WHI1660, WHI1086, WHI1249, and WHI1615 are human lymphoblastoid cell lines (kindly provided by Andrew Zinn) derived by transformation with Epstein-Barr virus and represent a normal female, a normal male, a female with XY chromosomes without Turner syndrome, and a female with XY chromosomes with Turner syndrome, respectively. Cells were grown in suspension (generation time, 26 h) as described previously (4) and harvested for polysomal or Western blot analyses at mid-log phase (5×10^5 to 7×10^5 cells/ml). P1798.C7 mouse lymphosarcoma cells were grown as suspension cultures and mitotically arrested by treatment with 0.1 μ M dexamethasone (Sigma) for 24 h as previously described (4).

Polysomal fractionation and RNA analysis. Polysomal fractionation and RNA analysis were performed as previously described (58).

Molecular probes. The isolated fragment probes used in the Northern blot analysis were a 0.97-kb fragment bearing the rpl32 processed gene, 4A (16); a 1.15-kb *Pst*I fragment containing mouse α -actin cDNA (39); a 1.8-kb *Bgl*II fragment containing mouse EF1 α cDNA (kindly provided by L. I. Slobin); a 0.8-kb *Hind*III fragment containing human growth hormone cDNA (kindly provided by T. Fogel, Bio-Technology General); a 0.74-kb *Nco*I-*Hind*III fragment containing green fluorescent protein (GFP) cDNA from pEGFP-C1 (Clontech); and a 0.6-kb *Eco*RI fragment containing the PKB α -specific probe (14).

Western blot analysis. Immunoblotting was performed as described previously (43) with anti-rpS6, anti-phospho-rpS6 (Ser235/236), or anti-phospho-rpS6 (Ser240/244), anti-PKB, anti-S6K1, anti-phospho-S6K1 (Thr389), and mouse-specific anti-phospho-4E-BP (Thr70) antibodies (Cell Signaling Technology, Beverly, Mass.). The preparation and specificities of the antibodies against rpS6 and its phosphorylated derivatives have been described (58). The use of longer gels in some experiments enabled the detection by anti-rpS6 of two bands, representing unphosphorylated and phosphorylated rpS6 (at least at positions Ser235/236), which were designated α and β , respectively.

RESULTS

TOP mRNAs are translationally activated by growth stimulation even without cell division. Cells double their ribosome content to ensure attainment of an appropriate size prior to division as well as survival of the daughter cells (1). Indeed, it has previously been shown that TOP mRNAs are translationally activated upon mitogenic stimulation (reference 37 and references therein). However, we were intrigued by the possibility that growth (increase in cell mass) induction might be sufficient for translational activation of TOP mRNAs even if not accompanied by cell division.

To directly address this issue, rat pheochromocytoma PC12 cells were serum starved for 72 h and then kept without serum, refed with serum, or treated with nerve growth factor (NGF)

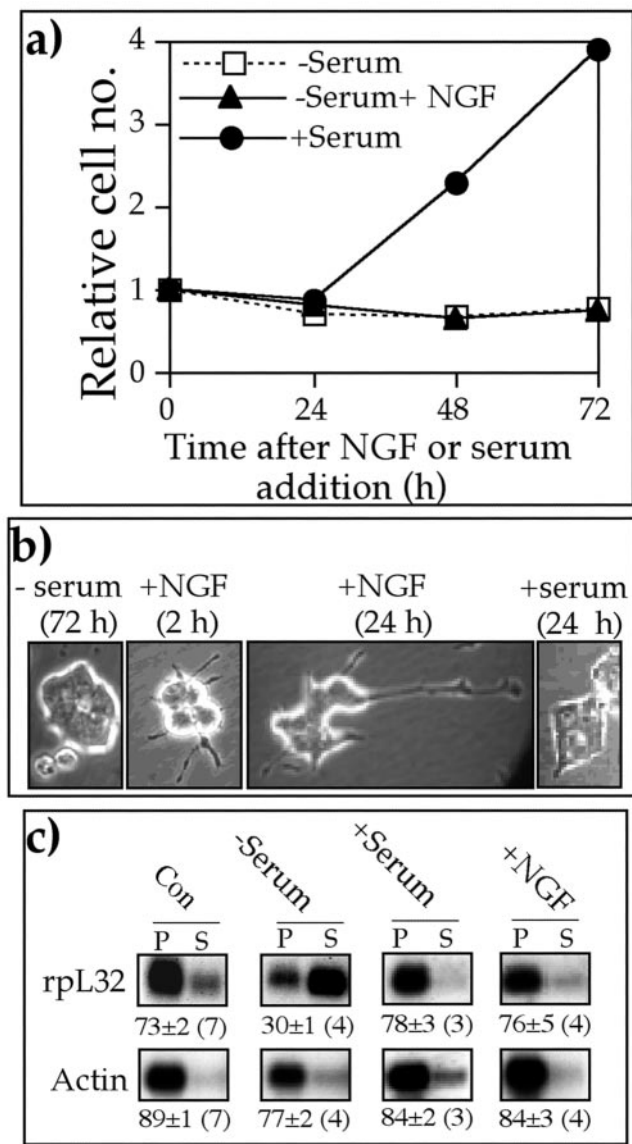


FIG. 1. NGF induces translational activation of TOP mRNAs in nondividing PC12 cells. (a) PC12 cells were serum starved for 72 h (zero time) and then either serum refeed (+Serum) or further serum starved without (-Serum) or with 25 nM NGF (-Serum + NGF). At the indicated times, cells were trypsinized and counted. Numbers of cells (average of at least two repetitions for each time point) were normalized to the number at time zero. (b) PC12 cells were serum starved for 72 h (-serum) and then either serum refeed (+serum) or treated with 25 nM NGF (+NGF). At the indicated times, cells were microscopically visualized at the same magnification. (c) Untreated (Con), 72-h serum-starved (-Serum), 72-h serum-starved and then 0.5-h serum-refed (+Serum), and NGF (25 nM)-treated (+NGF) PC12 cells were harvested, and cytoplasmic extracts were prepared. These extracts were centrifuged through sucrose gradients and separated into polysomal (P) and subpolysomal (S) fractions. RNA from equivalent aliquots of these fractions was analyzed by Northern blot hybridization with cDNAs for actin and rpL32. The radioactive signals were quantified by phosphorimager, and the relative translational efficiency of each mRNA is presented numerically beneath the autoradiograms as a percentage of the mRNA engaged in polysomes. These values are expressed as the average ± standard error of the mean, with the number of determinations shown in parentheses, or as the average, with the individual values in parentheses, if only two determinations were available.

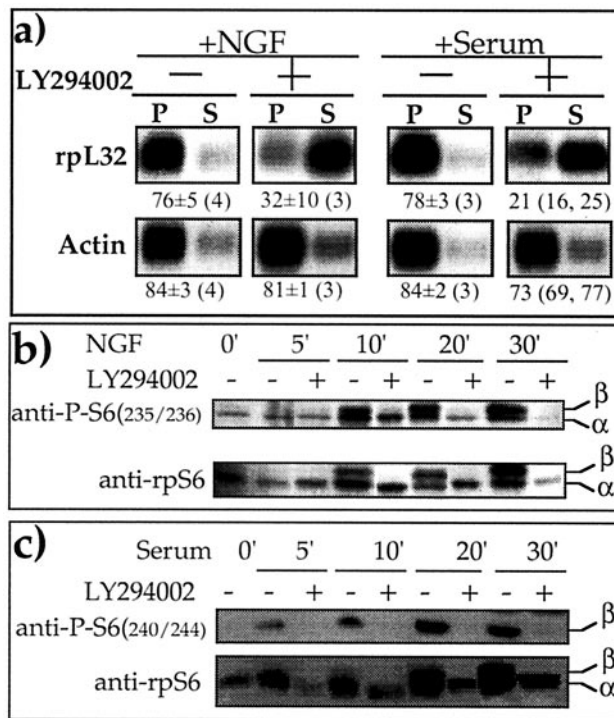


FIG. 2. LY294002 inhibits the phosphorylation of rpS6 as well as the translational activation of TOP mRNAs upon growth or mitogenic stimulation. (a) PC12 cells were serum starved for 72 h and then either treated with 50 ng of NGF per ml (+NGF) or serum refeed (+Serum) for 30 min without (-) or with (+) 50 μM LY294002. Subsequently, cells were harvested and subjected to polysomal analysis, as described in the legend to Fig. 1. (b and c) PC12 cells were serum starved for 72 h (0 min) and then either treated with 50 ng of NGF per ml (b) or serum refeed (c) without (-) or with (+) 50 μM LY294002 for the indicated times. The cytoplasmic proteins were subjected to Western blot analysis with the indicated antibodies. α and β represent hypophosphorylated and hyperphosphorylated forms of rpS6, respectively.

for an additional 72 h. Figure 1a shows, as reported previously (51), that cells resumed their proliferation only following serum refeeding. However, neurite outgrowth was readily detectable already 2 h after NGF introduction, with multiple long neurites apparent after 24 h (Fig. 1b). Polysomal analysis of a typical TOP mRNA encoding rpL32 showed that its translation was selectively repressed in serum-starved cells, as judged from its exclusion from polysomes. This mRNA, however, was rapidly (within 0.5 h) recruited into polysomes upon mitogenic or growth stimulation by serum refeeding or NGF treatment, respectively (Fig. 1c). It therefore appears that NGF-stimulated growth even without cell division suffices for translational activation of TOP mRNAs.

PI3-kinase-mediated pathway is indispensable for growth and mitotic activation of TOP mRNA translation. It has previously been shown that epidermal growth factor and NGF induce PI3-kinase in PC12 cells (11). Furthermore, we have demonstrated that translational control of TOP mRNAs by amino acid sufficiency requires the active PI3-kinase-dependent pathway (58). Hence, we set out to examine the role of PI3-kinase in translational activation of TOP mRNAs upon growth or mitogenic stimulation. Figure 2a shows that

LY294002, a specific inhibitor of PI3-kinase (66), completely blocked the translational activation of rpL32 mRNA in NGF-treated and serum-refed PC12 cells (Fig. 2a). Furthermore, this agent acted in parallel to completely inhibit the phosphorylation of rpS6 (Fig. 2b and 2c).

To further establish the role of a PI3-kinase pathway in translational activation of TOP mRNA, we selected 293 cells because of their high transfection efficiency and the fact that the translation of their TOP mRNAs is as sensitive to LY294002 as demonstrated for PC12 cells (Fig. 3a). These cells were cotransfected with two plasmids coding for L32-GFP mRNA and L32(-1_{C->A})-GH mRNA. The former starts with the 5'TOP motif of mouse rpL32 mRNA, whereas an extra A residue precedes this motif in the latter, and therefore these mRNAs behave essentially as TOP and non-TOP mRNAs, respectively (4, 58).

First, we examined the effect of interference in the delivery of signals along the pathway by overexpression of each of the following proteins: (i) p85 Δ iSH2-N, a dominant-negative mutant of the PI3-kinase regulatory subunit p85 which lacks the binding site for the catalytic subunit, p110 (50); (ii) pSG5L-HA-PTEN, which encodes hemagglutinin (HA)-tagged PTEN (48); (iii) PDK1^{K114G}, a dominant-negative mutant of PDK1 (18); and (iv) pAAA-PKB, a kinase-inactive, phosphorylation-deficient PKB α construct, with the mutations K179A, T308A, and S473A (68). Evidently, each of these overproduced proteins was sufficient to block the translational activation of L32-GFP mRNA in serum-refed cells (Fig. 3b). The apparent suppression of L32-GFP mRNA appeared to be selective for the 5'TOP-containing mRNA, as L32(-1_{C->A})-GH mRNA remained mostly associated with polysomes, although slightly repressed, in the presence of any of the expression vectors examined (Fig. 3b). It is therefore likely that PI3-kinase, PDK1, and PKB are necessary for the serum-induced recruitment of TOP mRNAs into polysomes.

In complementary experiments, we addressed the question of whether overexpression of constitutively active PI3-kinase or its effectors can rescue the translation of TOP mRNAs in serum-starved cells. Indeed, overexpression of p110*, a constitutively active mutant of PI3-kinase (24), or Gag-PKB α , a constitutively active PKB (9), alleviated the translational repression of L32-GFP mRNA exerted by serum starvation (compare p110* and pSG5-Gag-PKB α to empty vector and pSG5-Gag in Fig. 3c).

Mice deficient in PKB α are defective in both fetal and postnatal growth (12, 14). Likewise, we observed a slightly slower growth rate of PKB α ^{-/-} MEF relative to that of PKB α ^{+/+} MEF (generation times of \approx 42 h and 37 h, respectively). To examine whether this disparity in growth rate reflects differences in translation efficiency of TOP mRNAs, we set out to monitor the polysomal association of rpL32 mRNA. Figure 4a shows that despite the complete absence of PKB α mRNA, rpL32 mRNA was as efficiently recruited into polysomes upon serum stimulation of PKB α ^{-/-} MEF as of PKB α ^{+/+} MEF. Indeed, PKB α ^{-/-} MEF still expressed, albeit to a lesser extent, PKB (β and/or γ isoform), as demonstrated by Western blot analysis with anti-PKB antibody, which can detect all three isoforms (compare PKB to the Ponceau S-stained signals in Fig. 4b). Hence, the redundancy of the PKB activity or the action of other kinases with an overlapping specificity seems to

suffice for the requirement for normal translational control of TOP mRNAs, even in PKB α ^{-/-} MEF, and the mechanism underlying the slower growth of these cells has yet to be established.

Phosphorylation of rpS6 and S6K1 activity per se cannot support efficient translation of TOP mRNAs. The apparent inhibition of both the phosphorylation of rpS6 and the translational activation of TOP mRNAs by LY294002 (Fig. 2) is consistent with the common dogma, as yet unproved, that the two display a cause-and-effect relationships. To examine this notion, we monitored these two variables in lymphoblastoid cell lines. We have previously shown that the translation of a wide variety of TOP mRNAs is constitutively repressed in the proliferating WHI1249 line of human lymphoblastoid cells (3, 4). Here we show that this exceptional behavior appeared in all four lymphoblastoid cell lines (WHI1660, -1249, -1615, and -1086) examined, as judged by the predominant association of their rpL32 mRNAs with mRNP (mostly in the subpolysomal fraction in Fig. 5a). This commonality is underscored by the fact that these human cell lines were derived from four individuals having different genetic backgrounds.

The unique behavior of TOP mRNAs in lymphoblastoid cells is further exemplified by the fact that TOP mRNAs in mouse P1798 lymphosarcoma cells were efficiently translated in untreated cells and repressed only when mitotically arrested (Fig. 5a). Interestingly, translational repression in the lymphoblastoid cell lines occurred despite the apparent phosphorylation of rpS6, which was demonstrated by the phosphorylation-specific Ser240/244 antibody (Fig. 5b and 5c). It is noteworthy that rpS6 was also phosphorylated at Ser235/236 in the lymphoblastoid cells (see band β detected by anti-rpS6 in Fig. 5c). Furthermore, with a phosphorylation-specific antibody, we showed that S6K1 was phosphorylated at Thr389, a critical site for its activity (44), which suggests that the phosphorylation of rpS6 reflects S6K1 activity. These results therefore appear to support the notion that neither of these two suffices to enable efficient translation of TOP mRNAs.

Neither S6K1 activity nor rpS6 phosphorylation is required for translational activation of TOP mRNAs. The conflicting reports regarding causal relationships between S6K1 activity and the translational efficiency of TOP mRNAs in serum- or amino acid-stimulated cells (25, 58) prompted us to examine this issue with a mouse diploid ES cell line, p70^{S6K^{-/-}} cells, in which both alleles of S6K1 were disrupted by homologous recombination (28). Figure 6a shows that rpS6 in p70^{S6K^{-/-}} cells, unlike that of the parental cells (R1), was constitutively and selectively unphosphorylated (compare anti-phospho-rpS6 [Ser240/244] with anti-phospho-4E-BP [Thr70]). These results corroborate those originally obtained for these cells (28) but are inconsistent with a later report (33). Apparently, the unphosphorylated status of rpS6 does not diminish the efficient translation of TOP mRNAs when p70^{S6K^{-/-}} ES cells are mitotically active (see rpL32 in dividing cells, Fig. 6d). This observation therefore indicates that neither S6K1 activity nor rpS6 phosphorylation is necessary for efficient translation of TOP mRNAs.

It has previously been shown that mouse ES cells treated with retinoic acid are induced to differentiate into neuron-like cells (5). When p70^{S6K^{-/-}} ES cells were similarly treated, they exhibited a typical morphology of differentiating neurons, with

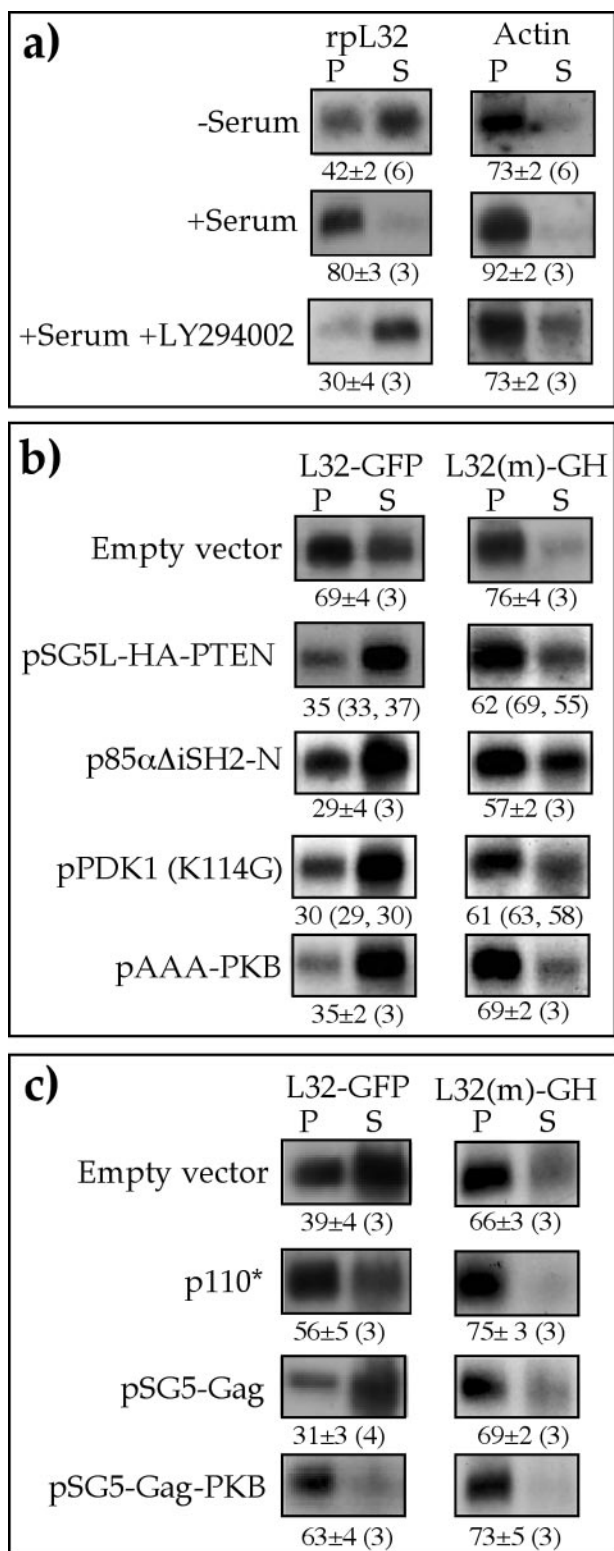


FIG. 3. PI3-kinase-dependent pathway mediates mitogenic signals into translational efficiency of TOP mRNAs. (a) 293 cells were serum starved for 26 h (-Serum) or serum re-fed for 3 h (+Serum) without or with 50 μ M LY294002, after which the cells were harvested. The polysomal distribution of mRNAs encoding rpL32 and actin was analyzed and presented as described in the legend to Fig. 1. (b and c) 293 cells were triply cotransfected with 2 μ g of vectors encoding L32-GFP and L32(-1_{C->A})-GH and 16 μ g of an empty vector (pSG5) or an

extensive neurite outgrowth (Fig. 6b). This differentiation was accompanied by a complete cessation of cell division (Fig. 6c), yet most of rpL32 was associated with polysomes, unlike the situation observed upon serum starvation (Fig. 6d). These results imply that translational control of TOP mRNAs by growth stimulation does not involve S6K1 activity.

Rapamycin inhibits growth- and mitogen-induced rpS6 phosphorylation, with only a minor repressive effect on translational activation of TOP mRNAs. A putative PKB phosphorylation site (S2448) was observed to be phosphorylated on mTOR in vivo, yet its role in the regulation of mTOR activity is still unclear (reference 49 and references therein). This observation, together with the conflicting reports concerning the ability of rapamycin to inhibit translational activation of TOP mRNAs (reference 23 and references therein) led us to examine whether mTOR is involved in signaling to TOP mRNAs upon growth or mitogenic stimulation.

To this end, we monitored the effect of rapamycin on the phosphorylation status of rpS6 and S6K1 as well as the polysome association of rpL32 mRNA in NGF-treated and serum-refed PC12 cells. Figures 7a and 7b show that phosphorylation of rpS6 was induced within 10 min after NGF introduction or serum refeeding. It has previously been shown that rapamycin completely abolished S6K1 activation in NGF-treated PC12 cell (29) and that its inhibitory effect on S6K1 activity was very rapid, with a half-time of about 2 min (15). Indeed, inhibition of the phosphorylation of Ser235/236 (Fig. 7a and 7b) and Ser 240/244 (Fig. 7c) by rapamycin was apparent at the earliest time points measured following NGF treatment and serum refeeding (10 min and 30 min, respectively). This inhibition reflects a block by rapamycin of S6K1 activation, as is evident from the phosphorylation status of its Thr389 (data not shown).

Nonetheless, despite this efficient inhibition, rapamycin had only a minor repressive effect on the translational activation of rpL32 mRNA. Thus, the proportion of rpL32 mRNA associated with polysomes increased from 30% in serum-starved cells (Fig. 1b) to 82% and 67% following 1 h of NGF treatment without and with rapamycin, respectively (Fig. 7d). Similarly, 83% and 78% of rpL32 mRNA were associated with polysomes 1 h after serum refeeding without and with rapamycin, respectively (Fig. 7d). Our results therefore show that mTOR inhibition cannot prevent translational activation of TOP mRNAs, as monitored within 60 min after NGF or serum addition.

It can be argued, yet with very low likelihood, that PC12 cells, being an established cell line, may have acquired abnormalities in translational control of TOP mRNAs that have effectively rendered it rapamycin resistant. To examine this possibility, we measured the effect of rapamycin on the translation of TOP mRNAs in serum-refed p70^{S6K-/-} ES cells, which are murine diploid cells. The sensitivity of mTOR to rapamycin in these cells was exemplified by the suppressed phosphorylation of 4E-BP, a direct substrate of mTOR (21),

expression vector, as indicated at the left. Then, 24 h later, cells were serum starved for 26 h and re-fed for 3 h (b) or serum starved for 26 h without refeeding (c). The polysomal distribution of L32-GFP and L32(-1_{C->A})-GH mRNAs was analyzed and presented as described in the legend to Fig. 1.

which was nearly complete just 10 min after addition of rapamycin (Fig. 7e), yet translation of two TOP mRNAs, encoding rpL32 and eEF1A, was only mildly repressed after 3 h of rapamycin treatment (Fig. 7f).

It has previously been shown for some cell lines that translational activation of TOP mRNAs was efficiently blocked if rapamycin was present during mitogenic stimulations which lasted up to 4 h (28, 56, 59, 60). Other cell lines, however, exhibited complete (26, 28) or nearly complete (25) resistance to such treatment. In light of these conflicting results, we set out to monitor the effect of rapamycin on proliferating 293 cells for increasing durations of time. Figure 8a shows that rpS6 in 293 cells was completely dephosphorylated at Ser235/236 (see band β in the upper panel of Fig. 8a) and Ser 240/244 (lower panel in Fig. 8a) within 30 to 60 min following exposure to rapamycin. Nevertheless, most ($60 \pm 2\%$ [$n = 3$]) of the pL32 mRNA in these cells remained associated with polyosomes even 8 h after the addition of rapamycin (Fig. 8b). Likewise, rapamycin induced a rapid dephosphorylation (within 30 min) of rpS6 in PC12 cells, yet had only a minor effect on the translational efficiency of rpL32 mRNA (decreased from $73\% \pm 2\%$ [$n = 7$] to $67\% \pm 5\%$ [$n = 3$] in polyosomes within 8 h) (data not shown).

The discrepancy between the fast inhibitory effect of rapamycin on the phosphorylation of rpS6 and the relative prolonged resistance of TOP mRNAs was highlighted by the differential response of the latter to rapamycin and LY294002 treatment. Thus, while 0.5 h of treatment of 293 cells with LY294002 led to selective and pronounced repression of mRNAs encoding rpL32 and eEF1A, 4 h of rapamycin treatment had only a mild inhibitory effect on these mRNAs (Fig. 8c). Nevertheless, this relative rapamycin resistance cannot be attributed to residual S6K1 activity, since 1 h of rapamycin treatment completely abolished S6K1 activity, as judged by the dephosphorylation of its Thr389 (Fig. 8d). These results therefore imply that the mTOR pathway does not have a direct role in regulating the translational efficiency of TOP mRNAs.

Clearly, even though rpS6 remained slightly phosphorylated in LY294002-treated cells (see band β in Fig. 8d), it was not sufficient to protect TOP mRNAs from rapid translational repression. Notably, the relative rapamycin resistance of TOP mRNAs, despite the complete inhibition of S6K1, also refutes causal relationships between the translational repression of TOP mRNAs and the inactivation of S6K1 (Fig. 8d), which are exerted by LY294002. In summary, Fig. 7 and 8 indicate that the mTOR pathway plays a minor role, if any, in the translational control of TOP mRNAs in all three cell lines examined (PC12, p70S6K^{-/-} ES, and 293), which represent three mammalian species (rat, mouse, and human, respectively) as well as different tissue origins and ploidy status.

DISCUSSION

Translational regulation of TOP mRNAs by growth and other signals. Previous studies have shown that mitogenic arrest at the G₀/G₁, S, or M phase of the cell cycle by a wide variety of means leads to translational repression of TOP mRNAs (37). In an attempt to identify a common denominator for all these stimuli, we reasoned that arresting cells at various

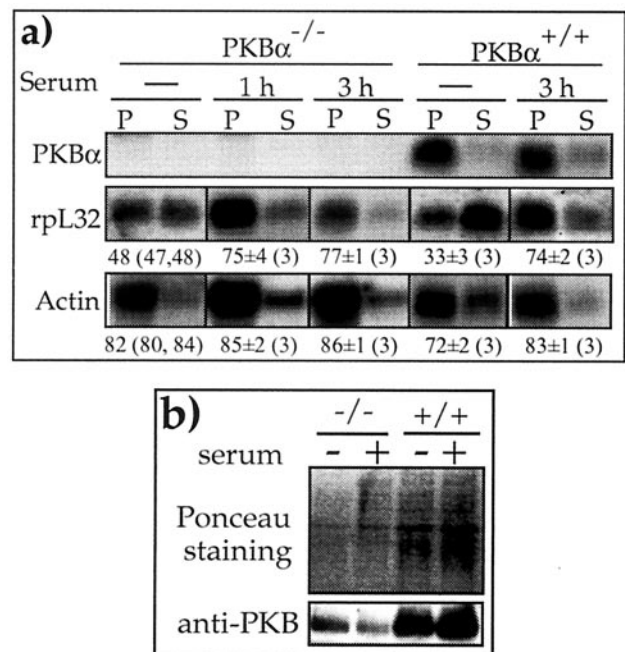


FIG. 4. Translation of TOP mRNAs is normally regulated in PKB α ^{-/-} MEF cells. (a) PKB α ^{+/+} and PKB α ^{-/-} MEF were harvested after being serum starved for 44 h without or with 1 or 3 h of serum refeeding. The polysomal distribution of the mRNAs encoding PKB α , rpL32, and actin was analyzed and presented as described in the legend to Fig. 1. (b) Cytoplasmic proteins from PKB α ^{+/+} (+/+) and PKB α ^{-/-} (-/-) MEF were harvested after being serum starved for 44 h without (-) or with 1 h of serum refeeding (+) and subjected to Western blot analysis. Bottom panel, immunoblotting with anti-PKB. Top panel, Ponceau S-stained membrane, showing the relative protein loading among samples. Note that the three PKB isoforms contain similar numbers of amino acids (from 466 to 481 residues).

stages along the cell cycle should also block their ability to progress into G₁ or G₂, where growth occurs. Hence, we have been intrigued by the question of whether it is the ability of cells to grow, rather than their ability to progress through the cell cycle, which matters for the translational control of TOP mRNAs. Indeed, our present results clearly show that PC12 cells can resume the translation of TOP mRNAs when they are induced to grow by NGF even if they remain mitotically arrested in the absence of serum (Fig. 1).

It therefore appears that cells are programmed to restrain the production of the translational apparatus, unless doubling its amount is required during cell divisions or for supporting the extensive protein synthesis needed upon growth stimulation. Likewise, cells limit the synthesis of their translational apparatus by repressing the translation of TOP mRNAs when they sense a shortage in the amino acid supply (58). It is worth noting that inducing cell cycle arrest by inhibiting DNA synthesis with aphidicolin or hydroxyurea also led to increased cell size (17, 19). Nonetheless, such treatments also resulted in repressed translation of TOP mRNAs (3, 4, 55). It is therefore important to determine whether the enlarged cell volume which is induced by inhibitors of the cell cycle involves an elevation in protein content or simply reflects swelling due to increased intracellular osmolarity.

TOP mRNAs are not confined to vertebrates, as many of the

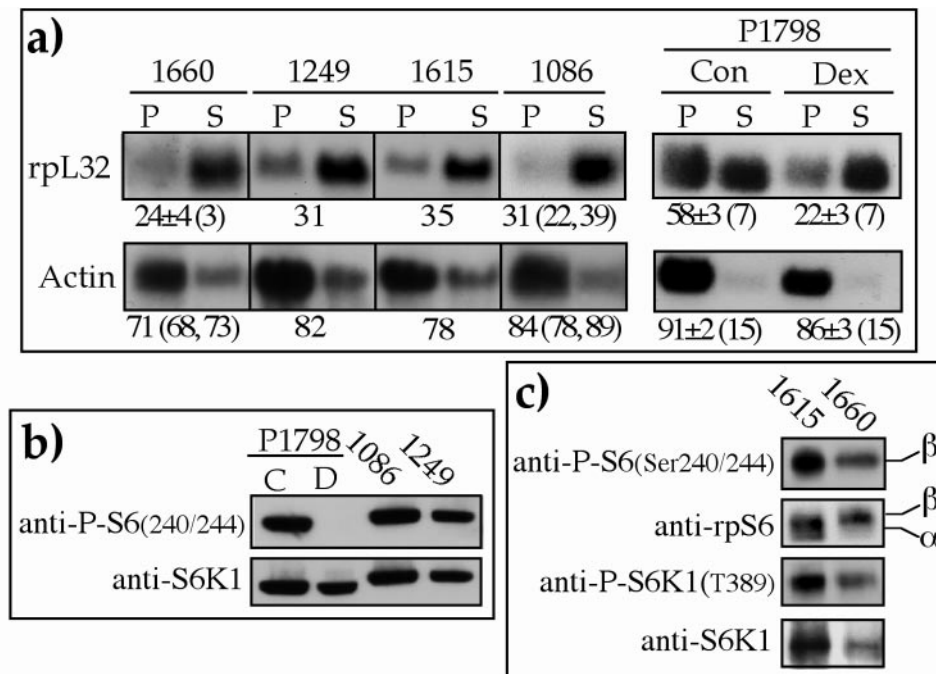


FIG. 5. Phosphorylation of rpS6 is not sufficient to enable efficient translation of TOP mRNAs. (a) Cytoplasmic extracts were prepared from four proliferating lymphoblastoid cell lines (WHI1660, WHI1249, WHI1615, and WHI1860) or from P1798 cells which were either dividing (Con or C) or nondividing due to 24 h of dexamethasone treatment (Dex or D). The polysomal distribution of the mRNAs encoding rpL32 and actin was analyzed and presented as described in the legend to Fig. 1. (b and c) Cytoplasmic proteins from the cells mentioned in panel a were subjected to Western blot analysis with the indicated antibodies.

ribosomal protein mRNAs in *Drosophila melanogaster* have been shown to contain the 5' TOP motif and to be translationally controlled (reviewed in reference 36). Interestingly, copulation in the fly leads to an abrupt elevation in the synthesis rate of ribosomal proteins in the paragonial gland, which is not accompanied by increased steady-state levels of the corresponding mRNAs or mitogenic activity. This observation suggests that translation of ribosomal protein mRNAs is activated after copulation to augment the protein synthesis capacity and thus to enable rapid replenishment of the secreted proteins (54). It therefore appears that enhanced translation of TOP mRNAs is attained under any physiological condition in which extensive protein synthesis is necessary (increase in cell mass, secretion, etc.).

It has previously been proposed that efficient translation of TOP mRNAs upon mitogenic stimulation plays a regulatory role in halting cell cycle progression until the growth response is completed (57, 61). However, the continuous proliferation of lymphoblastoid cells despite the constitutively repressed translation of TOP mRNAs (Fig. 5) (3, 4) indicates that efficient translation of these mRNAs is not a determinant in the control of the cell cycle and is not a prerequisite for cell division. Nevertheless, we cannot exclude the possibility that the synthesis of TOP mRNA-encoded proteins is still as efficient in lymphoblastoid cells as in any other dividing cells because of a compensatory increase in the abundance of the respective mRNAs, for example. Finally, translation of TOP mRNA is repressed upon inhibition of DNA synthesis by aphidicolin or prevention of chromosomal segregation by nocodazole even in the presence of a sufficient supply of growth factors and nutri-

ents. This implies that the translation of these mRNAs responds primarily to the ability of cells to progress through the cell cycle rather than to external mitogenic or nutritional cues.

Translational activation of TOP mRNAs is fully dependent on the PI3-kinase-mediated pathway. When cells are stimulated by growth factors, they display a wide variety of responses, including increased protein synthesis (2). A well-documented pathway which transduces mitogenic or growth signals involves PI3-kinase and its downstream effector, PKB (32). However, the role of this pathway in activation of protein synthesis has only been established for the activation of initiation factor 2B (eIF2B). Thus, eIF2B is inhibited through its phosphorylation by glycogen synthase 3 and is activated when the latter is phosphorylated, and consequently inactivated, by PKB (69).

In the present report, we demonstrate that both mitogenic and growth stimuli selectively activate the translation of TOP mRNAs in a PI3-kinase-PKB pathway-dependent manner. Thus, blocking the signal through this pathway by LY294002 completely abolished the translational activation of TOP mRNAs by growth or mitogenic signals (Fig. 2a and 3a). Translational activation of a chimeric TOP mRNA was similarly suppressed by overexpression of dominant-negative mutants of PI3-kinase, PDK1, and PKB α as well as overexpression of PTEN (Fig. 3b). It is conceivable, therefore, that the apparent enhanced protein synthesis in growth factor-stimulated cells reflects, at least partially, the activated translation of TOP mRNAs whose products comprise crucial components of the translational apparatus.

It is noteworthy that we have previously shown that a func-

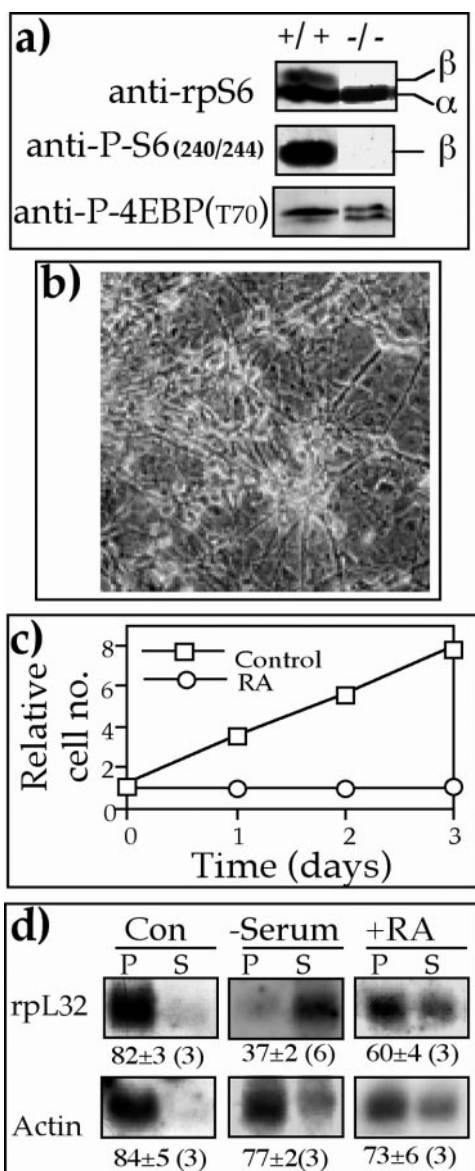


FIG. 6. Retinoic acid induces translational activation of TOP mRNAs in growing but not dividing $p70^{S6K-/-}$ ES cells. (a) Cytoplasmic proteins from wild-type ES cells (+/+) and $p70^{S6K-/-}$ cells (-/-) were subjected to Western blot analysis with the indicated antibodies. (b) $p70^{S6K-/-}$ ES cells were induced to neuronal differentiation (with retinoic acid), as described in Materials and Methods and visualized microscopically. (c) $p70^{S6K-/-}$ ES cells were either untreated (Con) or induced to neuronal differentiation with retinoic acid (RA), as described in Materials and Methods. Proliferation was monitored by measuring the A_{650} of the methylene blue dye extracted from stained cells. The absorbance measured 24 h after plating was arbitrarily set at 1, and absorbance measured at later time points (average of at least two repetitions for each time point) was normalized to that value. (d) $p70^{S6K-/-}$ cells were untreated (Con), serum starved for 40 h (-Serum), or induced to neuronal differentiation with retinoic acid (+RA). The polysomal distribution of the mRNAs encoding rpL32 and actin was analyzed and presented as described in the legend to Fig. 1.

tional PI3-kinase-mediated pathway is essential for translational activation of TOP mRNAs upon amino acids refeeding, even though the activity of neither PI3-kinase nor PKB is compromised by amino acid starvation (58). Finally, the fact

that translational repression of TOP mRNAs in serum-starved cells can be only partially relieved by overexpression of constitutively active PI3-kinase or PKB α (Fig. 3c) might reflect insufficient expression of these mutants. Alternatively, the PI3-kinase-mediated pathway is essential for this mode of regulation but is not sufficient.

mTOR-dependent pathway plays a minor role, if any, in translational control of TOP mRNAs. Activation of S6K and consequently the phosphorylation of rpS6, on the one hand, and translational activation of TOP mRNAs, on the other hand, are apparent under a variety of physiological conditions (35, 58). This tight correlation has laid the foundation for an attractive model according to which rpS6 phosphorylation is a prerequisite for efficient translation of TOP mRNAs. Nevertheless, several lines of evidence, presented in this and other recent reports, indicate that these two variables do not maintain cause-and-effect relationships. Thus, the translation of TOP mRNAs is constitutively repressed in dividing lymphoblastoid cells even though their rpS6 is phosphorylated (Fig. 5). Similarly, overexpression of RSK2 (a nonphysiological S6 kinase) and calyculin A (phosphatase inhibitor) treatment of 293 cells maintained rpS6 in its phosphorylated form, yet they failed to derepress TOP mRNAs upon amino acid starvation. This set of observations implies that rpS6 phosphorylation is not sufficient for recruitment of TOP mRNAs into polysomes.

Finally, TOP mRNAs are efficiently translated in $p70^{S6K-/-}$ ES cells (Fig. 6) and in mouse erythroleukemia cells (7) even though their rpS6 is constitutively unphosphorylated or dephosphorylated, respectively, indicating that rpS6 phosphorylation is not necessary either for efficient translation of TOP mRNAs or for cell division. Moreover, conditional disruption of rpS6 gene in the adult mouse liver did not inhibit hypertrophy of the liver in response to refeeding (67), indicating that not only its phosphorylation status but the entire rpS6 protein is dispensable for cellular growth.

It can be argued that it is the requirement for S6K1 activity, rather than just rpS6 phosphorylation, which is involved in the translational control of TOP mRNAs. However, several lines of evidence are inconsistent with this notion. (i) Elimination of S6K1 by knockout of the corresponding gene had no effect on translational activation of TOP mRNAs upon serum refeeding (Fig. 7f), induction for growth without cell division (Fig. 6), or amino acid replenishment (58). (ii) Mistargeting upstream signals by overexpression of $p70^{S6K, K123 M}$, a kinase-dead S6K1 mutant, in 293 cells completely abolished any S6K activity, as was evident by the unphosphorylated state of rpS6 (58). Nonetheless, efficient translation of TOP mRNAs was apparent in these cells when they were provided with complete growth medium or refeed with amino acids (58). Unlike the lack of inhibitory effect of this mutant, Jefferies and colleagues (25), who used another dominant-interfering mutant, $p70^{S6K A229}$, reported that its expression led to a modest suppression of the translational activation of a TOP mRNA following mitogenic stimulation. The difference between these results might reflect the number of repetitions used to obtain them (the latter was based on a single-repetition experiment).

(iii) Rapamycin completely inhibits S6K1 and rpS6 phosphorylation (15, 46), yet it has only a minor or no repressive effect on the translational activation of TOP mRNAs following serum or amino acid refeeding (Fig. 7) (25, 27, 28, 58) or in

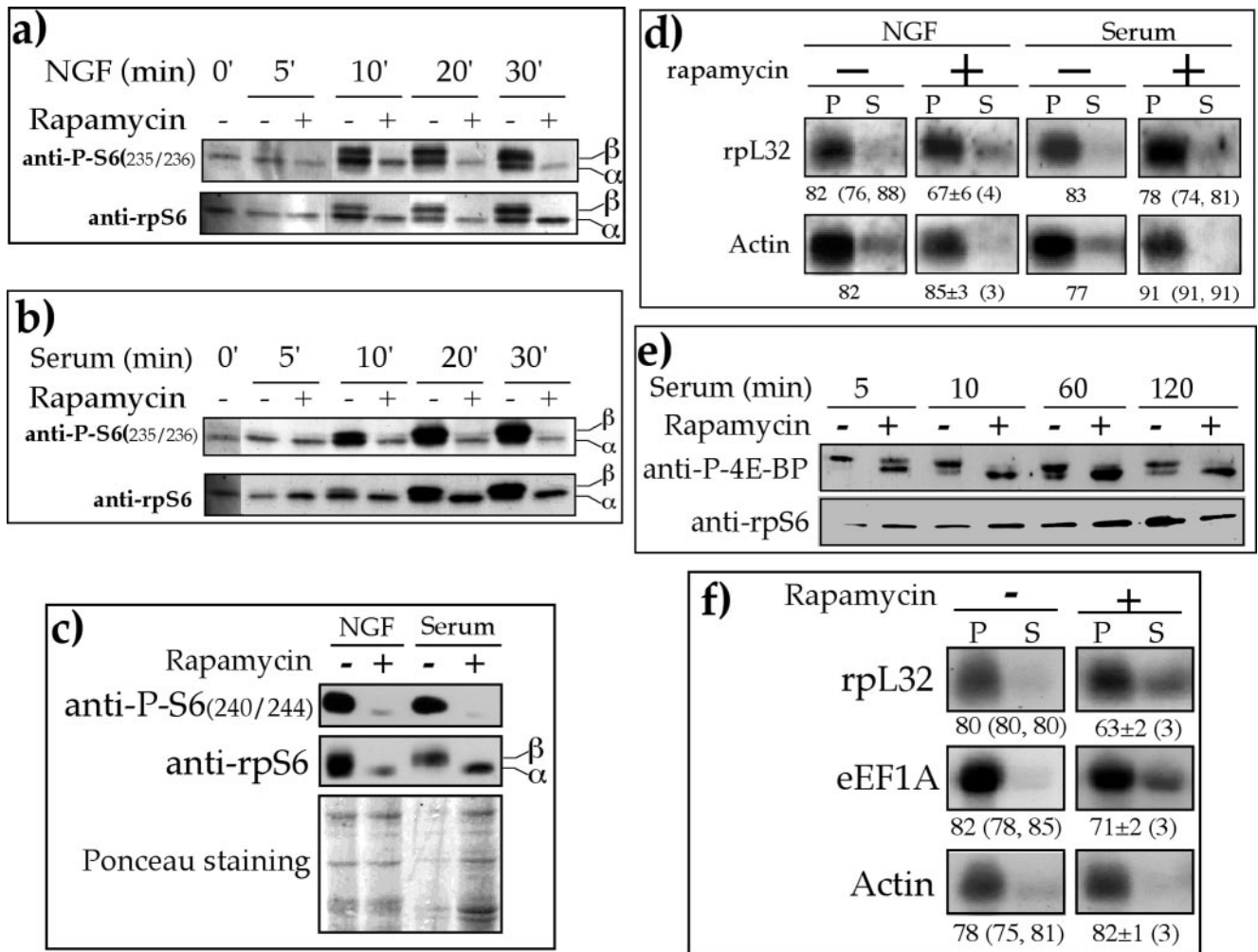


FIG. 7. Rapamycin has a minor and delayed inhibitory effect on the translation of TOP mRNAs. (a, b, and c) PC12 cells were serum starved for 72 h (0 min) and then either treated with 50 ng of NGF per ml (a and c) or serum refed (b and c) without (–) or with (+) 20 nM rapamycin for the indicated times (a and b) or for 30 min (c), after which the cells were harvested. The cytoplasmic proteins were subjected to Western blot analysis with the indicated antibodies. The bottom panel in c is the Ponceau S-stained membrane, showing the relative protein loading among samples. (d) PC12 cells were serum starved for 72 h and then either treated with 50 ng of NGF per ml or serum refed without (–) or with (+) 20 nM rapamycin for 60 min, after which they were harvested. The polysomal distribution of the mRNAs encoding rpL32 and actin was analyzed and presented as described in the legend to Fig. 1. (e) p70^{S6K}^{-/-} ES cells were serum starved for 40 h and then refed in the absence (–) or presence (+) of 20 nM rapamycin for the indicated times. Cytoplasmic proteins were subjected to Western blot analysis with the indicated antibodies. (f) p70^{S6K}^{-/-} ES cells were serum starved for 40 h and then refed for 3 h in the absence (–) or presence (+) of 20 nM rapamycin. The polysomal distribution of the mRNAs encoding rpL32, eEF1A, and actin was analyzed as described in the legend to Fig. 1. The results in panels d and f are expressed as the average \pm standard error of the mean, with the number of determinations in parentheses, or as the average, with the individual values in parentheses, if only two determinations were available.

proliferating cells of different genetic backgrounds (Fig. 8 and data not shown). It should be mentioned, however, that suppression of translational activation of TOP mRNAs by rapamycin has been shown upon mitogenic stimulation of MEF (56), lymphoblastoid cells (59), and T cells (60). Nevertheless, in the latter two cases, TOP mRNAs were constitutively repressed even without exposure to rapamycin, and therefore the significance of the effect of this drug is questionable. The minor inhibitory effect, if any, of rapamycin on the translation of TOP mRNAs in most cell lines examined so far is underscored by the rapid and complete repression of TOP mRNAs by amino acid starvation (within 1 h) ([58]) or LY294002 treatment (within 30 min) (Fig. 8c). Taken together, these data

clearly attest to the lack of any role of S6K1 and a minor role of mTOR in the translational control of TOP mRNAs in response to mitogenic, growth, or nutritional stimuli.

It has previously been suggested that the typical translational control of TOP mRNAs in p70^{S6K1}^{-/-} cells might be attributed to the compensating activity of S6K2 in these cells (56). However, two lines of evidence are inconsistent with the involvement of S6K2 in this mode of regulation. (i) Reports from several laboratories have shown that rapamycin, at the concentration used here (20 nM), also completely inhibits S6K2 in 293, mouse ES, and COS cells (30, 33, 52, 56), but has only a minor effect on translation of TOP mRNAs (58; this study). (ii) It has been shown that S6K2 is quite refractory to inhibition by

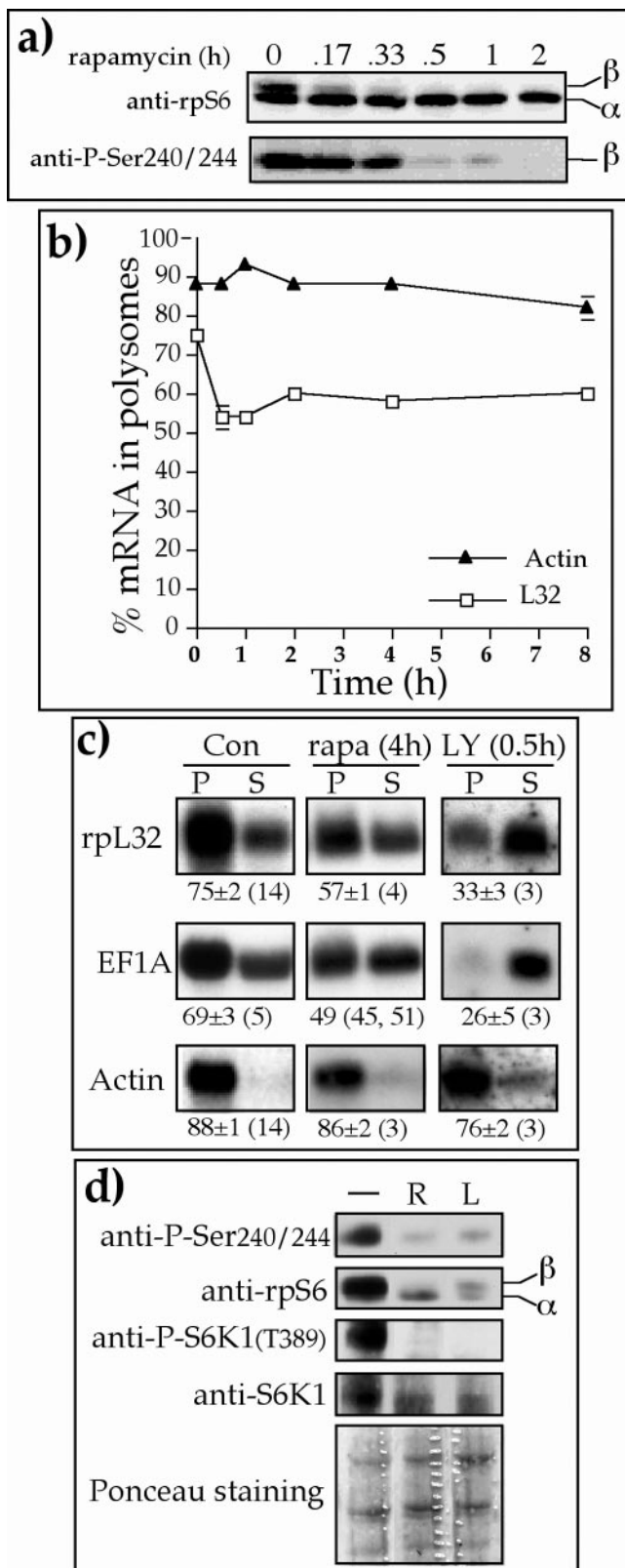


FIG. 8. Prolonged rapamycin treatment only mildly represses the translation of TOP mRNAs. (a and d) Proliferating 293 cells were treated with 20 nM rapamycin for the indicated time (a) or for 1 h (lane R in d) or with 50 mM LY294002 for 30 min (lane L in d), after which the cells were harvested. The cytoplasmic proteins were sub-

jected to Western blot analysis with the indicated antibodies. The bottom panel in d is the Ponceau S-stained membrane, showing the relative protein loading among samples. (b and c) Proliferating 293 cells were treated with 20 nM rapamycin for the indicated times (b) or for 4 h (c) or with 50 mM LY294002 for 30 min. The polysomal distribution of rpL32, eEF1A, and actin mRNAs was analyzed as described in the legend to Fig. 1. The relative amount of the mRNAs in polysomes is presented graphically in b and numerically in c.

amino acid withdrawal (38), yet this resistance was insufficient to prevent the repression of TOP mRNA translation upon amino acid starvation in 293 or p70^{S6K1-/-} cells (58). In any case, unequivocal negation of the role of S6K2 in the translational control of TOP mRNAs should await the establishment of S6K1/S6K2 double knockout mice or study of the translational control of TOP mRNAs in *dS6K*-null flies.

A tentative model depicting the signaling pathways leading to the translational activation of TOP mRNAs by mitogenic, growth, or amino acid sufficiency is presented in Fig. 9. According to this model, mitogenic and growth factors activate the PI3-kinase-dependent pathway, which transduces the respective signals through PDK1 and PKB into translational efficiency of TOP mRNAs. This pathway involves an unknown effector(s) (denoted as X). Signals from amino acids converge with the PI3-kinase-mediated pathway, and their transduction into TOP mRNAs is fully dependent on the integrity of this pathway. However, signaling from all external cues bifurcates prior to the mTOR step, as inferred from the ability of rapamycin to discern between the activity of mTOR and S6K on the one hand and the translational efficiency of TOP mRNAs on the other hand.

D. melanogaster might serve as an appropriate experimental system to resolve the role of the mTOR-dependent pathway in translational control of TOP mRNAs. The major advantage of this organism stems from the conservation of all five phosphorylatable serine residues in its rpS6 and the presence of a single S6K (*dS6K*) isoform (20). Homozygous flies null for *dS6K* were developmentally delayed to approximately half the size of wild-type flies. The dramatic reduction in size was due to smaller cells rather than decreased numbers of cells (40). Mutations in the *Drosophila* homologs of PI3-kinase, PKB, and mTOR have a striking effect on both cell number and cell size (reviewed in reference 31). Unfortunately, the translational efficiency of TOP mRNAs has never been addressed experimentally in any of these mutant flies or cells. Notably, a recent study demonstrated that *Drosophila* S6K controls cell growth in a *Drosophila* PKB- and *Drosophila* PI3-kinase-independent manner, implying that these enzymes reside on distinct pathways (47). If this conclusion is also applicable to mammalian cells, it is not surprising that we observed that translational efficiency of TOP mRNAs is affected by the PI3-kinase pathway in an S6K1-independent fashion.

Our present data and a previous report (58) refuted the only role primarily assigned for S6K and phosphorylated rpS6, translational control of TOP mRNAs. Hence, a new physiological role(s) for this extensively studied kinase should be established. Indeed, recent reports have implicated S6K1 in two other physiological processes, glucose homeostasis, through involvement in insulin secretion (45), and regulation of elongation factor 2 kinase (70) by its phosphorylation. Nev-

jected to Western blot analysis with the indicated antibodies. The bottom panel in d is the Ponceau S-stained membrane, showing the relative protein loading among samples. (b and c) Proliferating 293 cells were treated with 20 nM rapamycin for the indicated times (b) or for 4 h (c) or with 50 mM LY294002 for 30 min. The polysomal distribution of rpL32, eEF1A, and actin mRNAs was analyzed as described in the legend to Fig. 1. The relative amount of the mRNAs in polysomes is presented graphically in b and numerically in c.

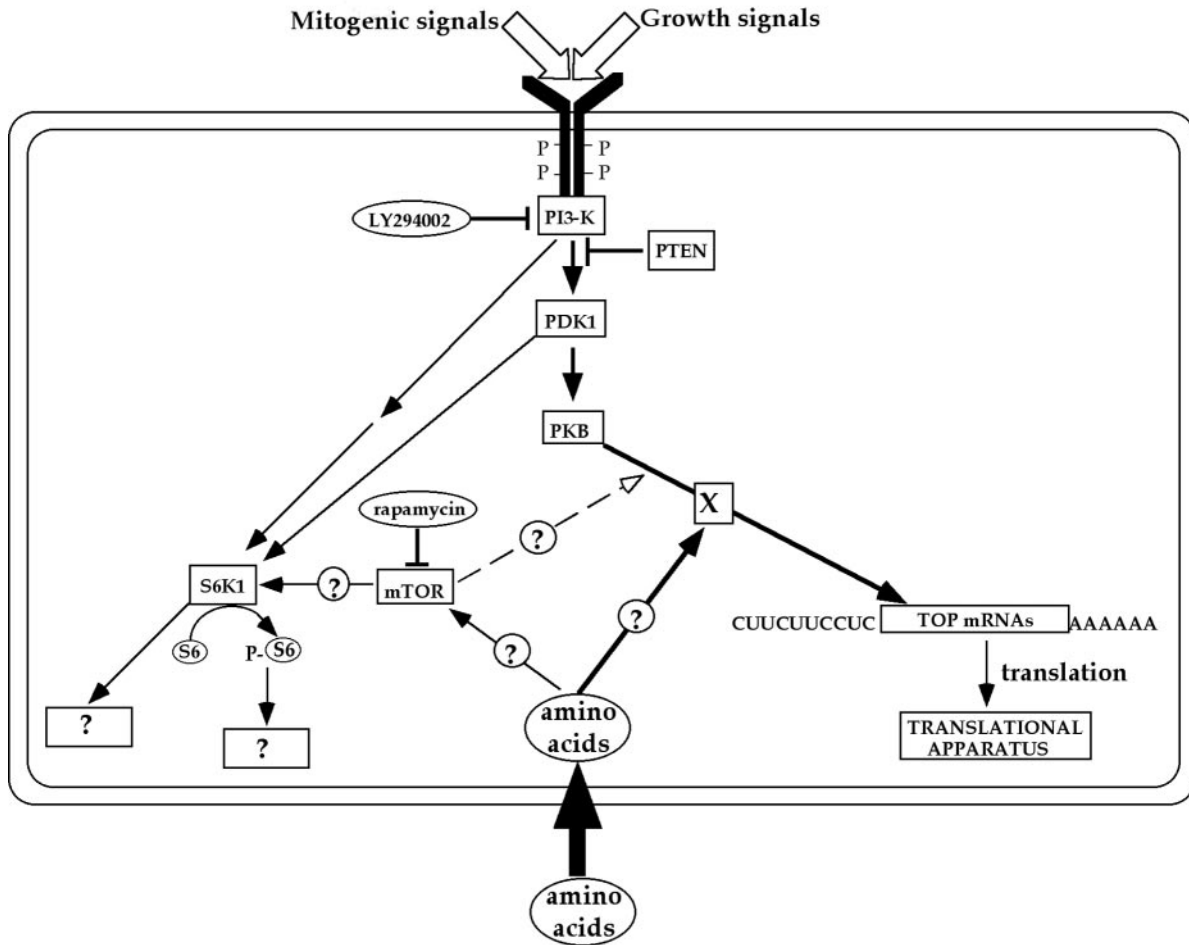


FIG. 9. Schematic representation of signal transduction pathways involved in activation of rpS6 phosphorylation and translational control of TOP mRNAs in serum- and amino acid-stimulated cells. Arrows delineate the flow of information. The open arrowhead represents the minor effect of rapamycin on TOP mRNA translation. The site of convergence of this effect with that of other signals is purely speculative. Circled and boxed question marks represent putative links and unknown targets, respectively. See text for details.

ertheless, the function of rpS6 phosphorylation still remains to be elucidated.

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