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Rapamycin suppresses 5′TOP mRNA translation through inhibition of p70^{s6k}

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Treatment of mammalian cells with the immunosuppressant rapamycin, a bacterial macrolide, selectively suppresses mitogen-induced translation of an essential class of mRNAs which contain an oligopyrimidine tract at their transcriptional start (5′TOP), most notably mRNAs encoding ribosomal proteins and elongation factors. In parallel, rapamycin blocks mitogen-induced p70 ribosomal protein S6 kinase (p70^{s6k}) phosphorylation and activation. Utilizing chimeric mRNA constructs containing either a wild-type or disrupted 5′TOP, we demonstrate that an intact polypyrimidine tract is required for rapamycin to elicit an inhibitory effect on the translation of these transcripts. In turn, a dominant-interfering p70^{s6k}, which selectively prevents p70^{s6k} activation by blocking phosphorylation of the rapamycin-sensitive sites, suppresses the translation of the chimeric mRNA containing the wild-type but not the disrupted 5′TOP. Conversion of the principal rapamycin-sensitive p70^{s6k} phosphorylation site, T389, to an acidic residue confers rapamycin resistance on the kinase and negates the inhibitory effects of the macrolide on 5′TOP mRNA translation in cells expressing this mutant. The results demonstrate that the rapamycin block of mitogen-induced 5′TOP mRNA translation is mediated through inhibition of p70^{s6k} activation.

Keywords: mitogenesis/p70^{s6k}/ribosome biogenesis/S6 phosphorylation/translational control

Introduction

The biogenesis of translational components plays an essential role in the growth and proliferation of cells in response to a mitogenic signal (Nasmyth, 1996). In the case of ribosomes, many of the protein and RNA constituents are structurally well characterized (Wool *et al.*, 1996); however, little is known concerning the mechanisms and signalling pathways responsible for regulating their coordinate expression. Recent studies have shown that the mRNA transcripts for all ribosomal proteins studied to date, as well as protein synthesis elongation factors, contain an unusual oligopyrimidine tract at their transcriptional start site, termed a 5′TOP, which confers translational control on their expression in response to mitogens (Jefferies and Thomas, 1996; Meyuhas *et al.*, 1996). The number of individual transcripts that make up the 5′TOP mRNA family is small; however, in abundance they can represent up to 20% of the total mRNA in the cell (Meyuhas *et al.*, 1996). Unlike most mammalian mRNAs, the first residue immediately following the cap site in the 5′TOP is invariably a cytosine, which is succeeded by a stretch of 5–14 pyrimidines, varying in length and composition, depending on the particular transcript. In growth-arrested cells, these mRNA transcripts are distributed between mRNP particles and small polysomes, made up largely of monosomes and disomes. Unlike most cellular mRNAs which redistribute to polysomes of the same size following mitogenic stimulation, 5′TOP mRNAs redistribute to polysomes of larger size, the mean distribution being dependent on the length of the transcript (Jefferies *et al.*, 1994b). This selective regulation of 5′TOP mRNAs appears to be dependent on the polypyrimidine tract as well as its location at the transcriptional start site (Meyuhas *et al.*, 1996). This conclusion is based on several observations; (i) fusing the 5′-untranslated region (5′UTR) of a 5′TOP mRNA to a non-5′TOP mRNA confers translational control on the chimeric transcript (Mariottini and Amaldi, 1990; Hammond *et al.*, 1991; Levy *et al.*, 1991), (ii) whereas placing the 5′TOP motif within the 5′UTR of a non-5′TOP mRNA does not confer translational control on the chimeric transcript (Levy *et al.*, 1991; Avni *et al.*, 1994), and (iii) substitution of a single purine in the +1 position for the cytosine abolishes translational control of the chimeric transcript (Levy *et al.*, 1991). Although little is known concerning the mechanism by which the acute translational up-regulation of 5′TOP mRNAs is controlled, recent studies have demonstrated that their expression is suppressed selectively by the immunosuppressant rapamycin, suggesting that the FRAP/p70^{s6k} signalling pathway is modulating this response (Jefferies *et al.*, 1994a).

Rapamycin is a bacterial macrolide which forms a gain-of-function inhibitory complex with the immunophilin FKBP12, targeting a large molecular weight protein termed FRAP or mTOR (Brown *et al.*, 1994; Sabatini *et al.*, 1994; Sabers *et al.*, 1995). Though mTOR/FRAP has homology to both lipid and protein kinases, the only allied activity identified to date is autophosphorylation (Brown *et al.*, 1995; Brunn *et al.*, 1996). Rapamycin treatment of cells also leads to the dephosphorylation and inactivation of the p70^{s6k} and, in parallel, the dephosphorylation of ribosomal protein S6 (Chung *et al.*, 1992; Jefferies *et al.*, 1994a). Furthermore, a point mutant of mTOR/FRAP, which fails to bind the rapamycin–FKBP12 inhibitory complex, when transiently co-transfected with p70^{s6k} protects the kinase from inactivation by the macrolide (Brown *et al.*, 1995). The latter studies lent support to the hypothesis that mTOR/FRAP is an upstream activator of

the p70^{s6k}. Since p70^{s6k} has been implicated in translation through S6 phosphorylation (Jefferies and Thomas, 1996), the effect of rapamycin on protein synthesis was examined. Rapamycin pre-treatment of mitogen-stimulated Swiss 3T3 cells caused only an ~10–15% inhibition of global protein synthesis; however, it severely repressed the recruitment of 5′TOP mRNAs into polysomes with no effect observed on the translation of the non-5′TOP-containing mRNAs coding for β-actin, protein synthesis initiation factor eIF-4A and β-tubulin (Jefferies *et al.*, 1994a). These studies led to the speculation that the p70^{s6k}, through S6 phosphorylation, is involved in the selective up-regulation of 5′TOP mRNAs. However, this model has been questioned by the recent observation that, in response to mitogens, the phosphorylation of at least three other translational components also are affected by rapamycin. None of these three proteins, including initiation factor eIF-4E (Mendez *et al.*, 1996), the eIF-4E repressor 4E-BP1 (Beretta *et al.*, 1996) and the kinase which modulates elongation factor eEF-2 phosphorylation, calcium calmodulin-dependent kinase III (Redpath *et al.*, 1996), are substrates for the p70^{s6k}.

The finding that mTOR/FRAP, based on the inhibitory effects of rapamycin, has a number of potential downstream translational targets has put into question the role of p70^{s6k} in regulating 5′TOP mRNA expression (Lin *et al.*, 1995; Proud, 1996). To address this issue further would require more precise tools than rapamycin. Such tools might be generated by utilizing recently described p70^{s6k} phosphorylation site mutants. Activation of p70^{s6k} is associated with two distinct sets of mitogen-induced phosphorylation sites. The first set of sites are flanked by a proline in the +1 position, exhibit rapamycin resistance, except for S411, and reside within an autoinhibitory domain whose function appears to modulate kinase activity (Han *et al.*, 1995; Mahalingam and Templeton, 1996). In contrast, the second set of sites are rapamycin sensitive and are flanked by large aromatic residues (Pearson *et al.*, 1995). Two of these sites, T229 in the activation loop and T389 in the conserved linker region, appear essential for kinase activity based on the finding that substitution of an alanine at either site ablates kinase activity (Pearson *et al.*, 1995). Kinase-dead mutants, harbouring a neutral residue in the phosphorylation site of the activation loop, in some instances have been shown to act as dominant-interfering mutants preventing signalling to downstream targets (Pagès *et al.*, 1993). Furthermore, T389, the principal target of rapamycin-induced p70^{s6k} dephosphorylation and inactivation, when converted to an acidic residue, confers rapamycin resistance on the kinase (Pearson *et al.*, 1995). When used in combination, these phosphorylation site mutants could be exploited to designate downstream functional targets.

To evaluate the model that rapamycin inhibits 5′TOP translation through blocking the ability of p70^{s6k} to signal to the polypyrimidine tract, it was first determined whether an intact polypyrimidine tract was required for the macrolide to exert its inhibitory effect on translational up-regulation of these mRNAs. Next, a potential dominant-interfering mutant of p70^{s6k} was examined for its effect on reporter p70^{s6k} activity as well as for its selectivity for this kinase. Finally, the dominant-interfering and rapamycin-resistant mutants were examined for their ability to either suppress mitogen-induced 5′TOP mRNA translation or to protect it from the inhibitory effects of rapamycin.

Results

Rapamycin requires intact 5′ TOP to exert an inhibitory effect

To address the importance of the 5′TOP motif in serving as the inhibitory target of rapamycin in suppressing the translation of 5′TOP mRNAs, advantage was taken of two NIH 3T3 cell lines stably expressing one of two chimeric mRNA constructs (Avni *et al.*, 1994). The wild-type chimeric mRNA contains the first 29 nucleotides from the 5′UTR of ribosomal protein S16, including the 5′TOP, fused to the human growth hormone (hGH) mRNA, whereas the mutant chimeric construct has five of the eight pyrimidines forming the 5′TOP replaced by purines (Figure 1A and B). In quiescent cells, the majority of wild-type S16-hGH transcript sediments with mRNP particles or as monosomes/disomes, whereas the addition of serum induces both populations to relocalize to polysomes containing 7–9 ribosomes per transcript (Figure 1C, left and middle panels, respectively). These results are very similar to those reported for elongation factor-1α (eEF-1α) and other 5′TOP mRNAs (Jefferies *et al.*, 1994a; Terada *et al.*, 1994). In contrast, disruption of the 5′TOP by insertion of the five purines abolishes translational regulation of the mutant cm5S16-hGH chimeric mRNA such that most of the transcript is now found associated with polysomes containing ~7–9 ribosomes per transcript, regardless of growth state (Figure 1C, compare left and middle panels). If serum-stimulated cells expressing the wild-type S16-hGH transcript were treated with rapamycin, little effect was observed on global translation based on polysome profiles (Figure 1C, compare middle and right panels); however, the wild-type chimeric transcript largely redistributes from polysomes to mRNP particles and monosomes/disomes (Figure1C, right panel). In striking contrast to the wild-type chimeric transcript, rapamycin treatment of stimulated cells expressing the mutant cm5S16-hGH mRNA had no measurable effect on message distribution, with the mRNA remaining associated with polysomes. In parallel, stimulation of either cell type leads to an increase in p70^{s6k} activity as compared with quiescent cells (Figure 1D, compare lanes 2 and 5 with 1 and 4, respectively), with the addition of rapamycin totally abolishing this response (Figure 1D, lanes 3 and 6). Although rapamycin abolishes p70^{s6k} activity, it only suppresses 5′TOP mRNA translation, suggesting that, if the effects of rapamycin are exerted through p70^{s6k}, there must be a rapamycin/p70^{s6k}-independent pathway involved in this response. Collectively, the results also demonstrate that an intact oligopyrimidine tract is required for rapamycin to exert its effect on the translation of 5′TOP mRNAs.

Effect of a dominant-interfering mutant on p70^{s6k} activation

A powerful tool in identifying upstream mediators of specific signalling pathways has been the use of dominant-interfering mutants. In the case of kinases, dominant-interfering mutants have been generated by mutating either the essential lysine in the conserved ATP-binding pocket

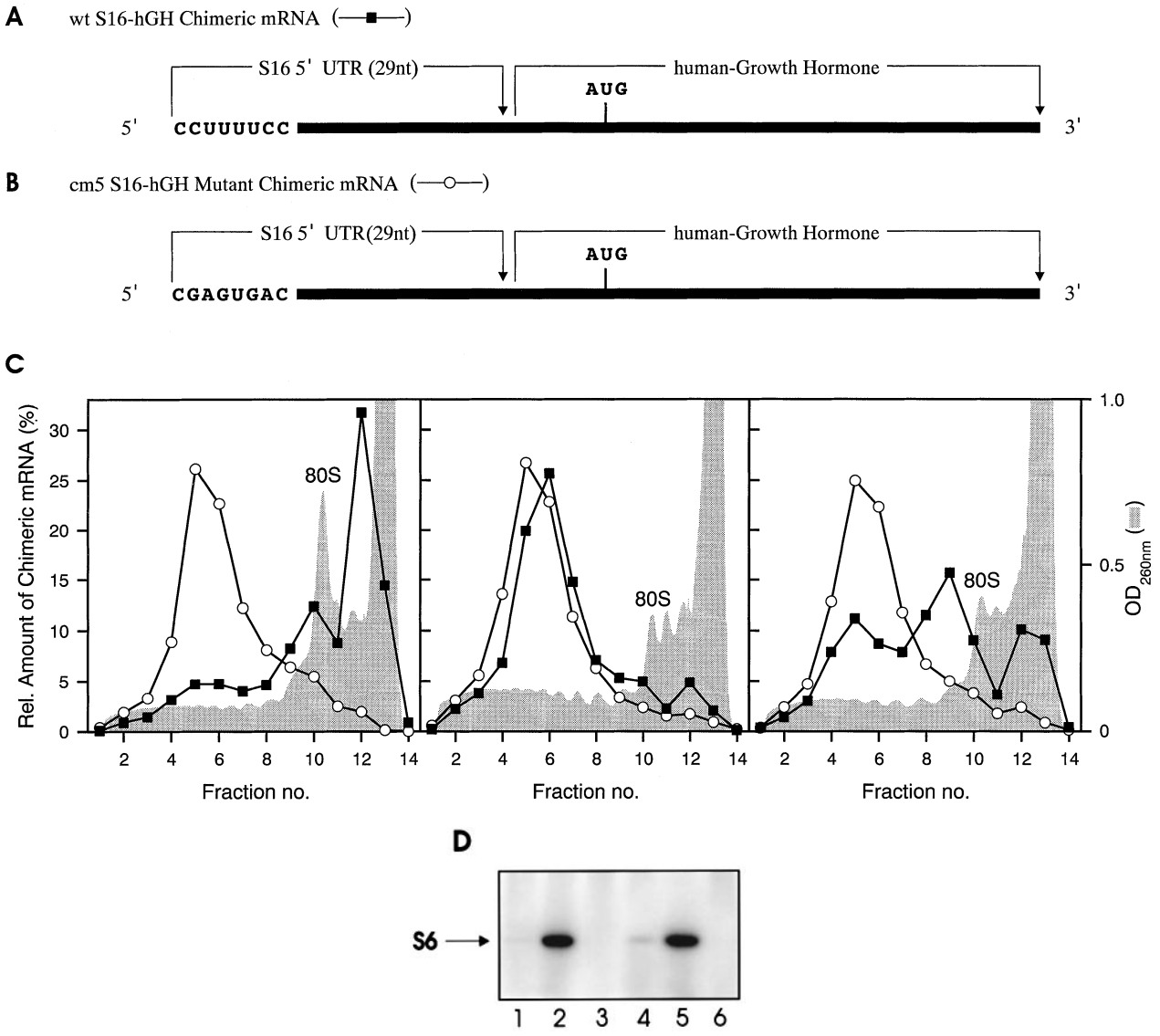

Fig. 1. Rapamycin suppresses translation by a mechanism requiring a 5′TOP. Two NIH 3T3 cell lines were used. (**A**) One cell line was stably transfected with a wtS16-hGH chimeric gene under the control of the S16 promoter which generated a chimeric mRNA where 29 nucleotides of the 5′UTR of S16 were joined to the hGH coding region. (**B**) The second cell line was stably transfected with a cm5S16-hGH mutant gene which generated an identical transcript except that five of the pyrimidines were mutated to purines. (**C**) Cytoplasmic extracts were prepared from the two NIH 3T3 cell lines either quiesced (left panel), stimulated with 10% FCS for 3 h (middle panel) or stimulated with FCS for 3 h and then treated for an additional 1 h with 20 nM rapamycin (right panel). wtS16-hGH chimeric mRNA is indicated by (■) and cm5S16-hGH chimeric mRNA by (○). The extracts were centrifuged on 17.1–40% linear sucrose gradients, fractionated and subjected to Northern blot analysis using a probe directed against the hGH portion of the chimeric transcripts. Blots were then developed with a PhosphorImager (Molecular Dynamics) and the signal from each gradient fraction was quantified using Imagequant software (Molecular Dynamics) and calculated as a percentage of the total signal from the 14 fractions. The shaded areas depict the $OD_{260\ nm}$ polysome gradient profile from the NIH 3T3 cells under the indicated conditions, and the position of the 80S peak is noted. (**D**) Immunocomplex assays of p70^{s6k} from the two NIH 3T3 cell lines expressing either wtS16-hGH chimera (lanes 1–3) or cm5S16-hGH chimera (4–6). Kinase activity was assayed from quiescent cells (lanes 1 and 4), cells stimulated with 10% FCS for 3 h (lanes 2 and 5) or cells stimulated with FCS for 3 h and then treated for an additional 1 h with 20 nM rapamycin (lanes 3 and 6). S6 in 40S ribosomal subunits was used as substrate.

(Sanchez *et al.*, 1994) or a critical phosphorylation site in the activation loop, such that kinase activity of the resulting construct is ablated (Pagès *et al.*, 1993). When transiently overexpressed, these mutants are thought to act by sequestering upstream activators, preventing the activation of the endogenous enzyme. Recently, T229 in the activation loop of p70^{s6k} was identified as a principal phosphorylation site associated with kinase activation (Pearson *et al.*, 1995; Weng *et al.*, 1995a). Since substitution of T229 by either an acidic or neutral residue ablated kinase activity (Pearson *et al.*, 1995; Weng *et al.*, 1995a), it was reasoned that transient overexpression of a kinase-inactive mutant, p70^{s6k}A229, might function as a dominant-interfering mutant. To test this possibility, a myc- and glutathione S-transferase (GST)-tagged p70^{s6k} reporter construct, myc-p70^{s6k}-GST, was co-transfected with increasing amounts of the myc epitope-tagged-p70^{s6k}A229 construct in human 293 cells. These cells were chosen because of their high transfection efficiency, and the fact that they regulate 5′TOP expression in a similar manner to that observed for 3T3 cells (see below). The results demonstrate that increasing amounts of transfected myc-p70^{s6k}A229

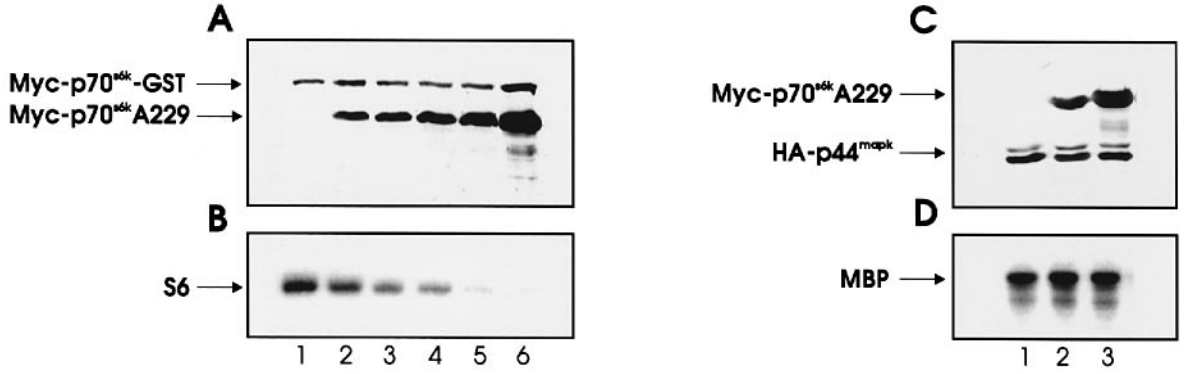

Fig. 2. Expression of dominant-interfering p70^{s6k}A229 blocks p70^{s6k}, but not p44^{mapk} activation. (A and B) 293 cells were transiently transfected using a calcium phosphate method with 1 μg of myc-p70^{s6k}-GST plasmid alone (lane 1) or co-transfected with increasing amounts of myc-p70^{s6k}A229 plasmid (lanes 2–6, 0.5, 1, 2, 5 and 10 μg of plasmid DNA respectively). Following 24 h serum starvation and 45 min of FCS (10%) treatment, cell extracts were prepared and equal amounts of total protein were resolved by SDS–PAGE and subjected to Western blot analysis. The membrane was probed with 9E10 antibody to analyse the expression level of the two myc epitope-tagged forms of p70^{s6k} and detected by fluorometry (**A**). (**B**) Reporter myc-p70^{s6k}-GST, was purified from cell extracts using glutathione–Sepharose and assayed for S6 kinase activity using 40S ribosomal subunits. (C and D) 293 cells were transiently transfected with 1 μg of HA-p44^{mapk} plasmid alone (lane 1) or co-transfected with 0.5 (lane 2) or 10 μg (lane 3) of myc-p70^{s6k}A229 plasmid. Cells were serum starved for 24 h and then stimulated for 10 min with FCS (10%). Extracts were analysed for HA-p44^{mapk} and myc-p70^{s6k}A229 protein expression on Western blots probed with a mix of 9E10 (myc epitope tag) and 12CA5 (HA tag) antibodies which were then detected by fluorometry (**C**) as described in Materials and methods. (**D**) HA-p44^{mapk} was immunoprecipitated with 12CA5 antibody and assayed for MBP kinase activity.

blocked reporter myc-p70^{s6k}-GST activation in a dose-dependent manner, as measured in an activity assay employing S6 as a substrate (Figure 2A and B). It should be noted that a similar dominant-interfering mutant has been obtained by altering the lysine in the ATP-binding pocket to glutamine (P.B.Dennis and G.Thomas, unpublished data). That the dominant-interfering effect of myc-p70^{s6k}A229 was specific for the p70^{s6k} was shown by the fact that its overexpression had no effect on the serum-induced activation of co-transfected haemagglutinin (HA)-tagged p44^{mapk} in an immune complex assay employing myelin basic protein (MBP) as a substrate (Figure 2C and D). The results also demonstrate that increased expression of the dominant-negative construct has no effect on the expression of either reporter construct, myc-p70^{s6k}-GST or HA-p44^{mapk} (Figure 2A and C, respectively). Thus the p70^{s6k}A229 construct acts as a selective inhibitor of the p70^{s6k} signal transduction pathway. Surprisingly, the amount of ^{32}P incorporated into myc-p70^{s6k}-GST in the absence and presence of myc-p70^{s6k}A229 was indistinguishable (data not shown), suggesting either that the inhibitory effect was not through an upstream kinase or that the phosphorylation of only a minor subset of sites was being affected by the dominant-interfering construct. To address this point, two-dimensional phosphopeptide maps were analysed of the reporter myc-p70^{s6k}-GST derived from either serum-stimulated cells co-expressing the empty vector or the dominant-interfering mutant myc-p70^{s6k}A229. As compared with expression of the reporter alone, co-expression in the presence of the dominant-interfering mutant blocked phosphorylation of T229, in the activation loop, as well as T389 and S404, in the linker region (see Figure 3A), and significantly reduced phosphorylation of S411 in the autoinhibitory domain (Figure 3B and C, respectively). The phosphorylation of T229 and T389 appears critical for kinase activation (Pearson *et al.*, 1995; Dennis *et al.*, 1996). In contrast, the remaining major phosphorylation sites, including S418, T421 and S424 in the autoinhibitory domain, are largely unaffected. Interestingly, the phosphorylation sites affected are identical to those blocked by rapamycin pre-treatment (Han *et al.*, 1995; Pearson *et al.*, 1995). It should be noted that the phosphopeptides migrating above the origin have been determined to be distinct from T229- and T389-containing phosphopeptides by converting both sites to serine and analysing individual peptides for phosphoamino acid content (data not shown). Thus, not only is the dominant-interfering construct specific for p70^{s6k}, but it selectively blocks the phosphorylation of the same subset of sites as rapamycin, most likely by competing for a proximal upstream activator of p70^{s6k}.

Effect of a dominant-interfering mutant on 5′ TOP mRNA translation

As the dominant-interfering myc-p70^{s6k}A229 construct specifically blocks p70^{s6k} activation, we reasoned that it would serve to discern whether the inhibitory effects of rapamycin on 5′TOP translation are through inhibition of the kinase. To test this possibility, 293 cells were co-transfected with cDNA constructs expressing either the wild-type or mutant S16-hGH chimeric mRNAs in combination with either the empty vector or the vector expressing the myc-p70^{s6k}A229 construct. The mutant cm5S16-hGH chimeric mRNA in the presence of empty vector was translated efficiently and associated with polysomes in both quiescent and serum-stimulated cells (Figure 4A), consistent with the results obtained in NIH 3T3 cells (Figure 1C left and middle panels). Furthermore, co-expression of dominant-negative myc-p70^{s6k}A229 did not affect the distribution of the mutant cm5S16-hGH chimeric mRNA in either quiescent or serum-stimulated cells (compare Figure 4A and B). In serum-deprived 293 cells, the transiently expressed wild-type S16-hGH chimeric mRNA co-expressed with the vector alone behaved in a similar manner to that observed under stable expression conditions in NIH 3T3 cells, except that more of the wild-type mRNA partitioned with the monosome/

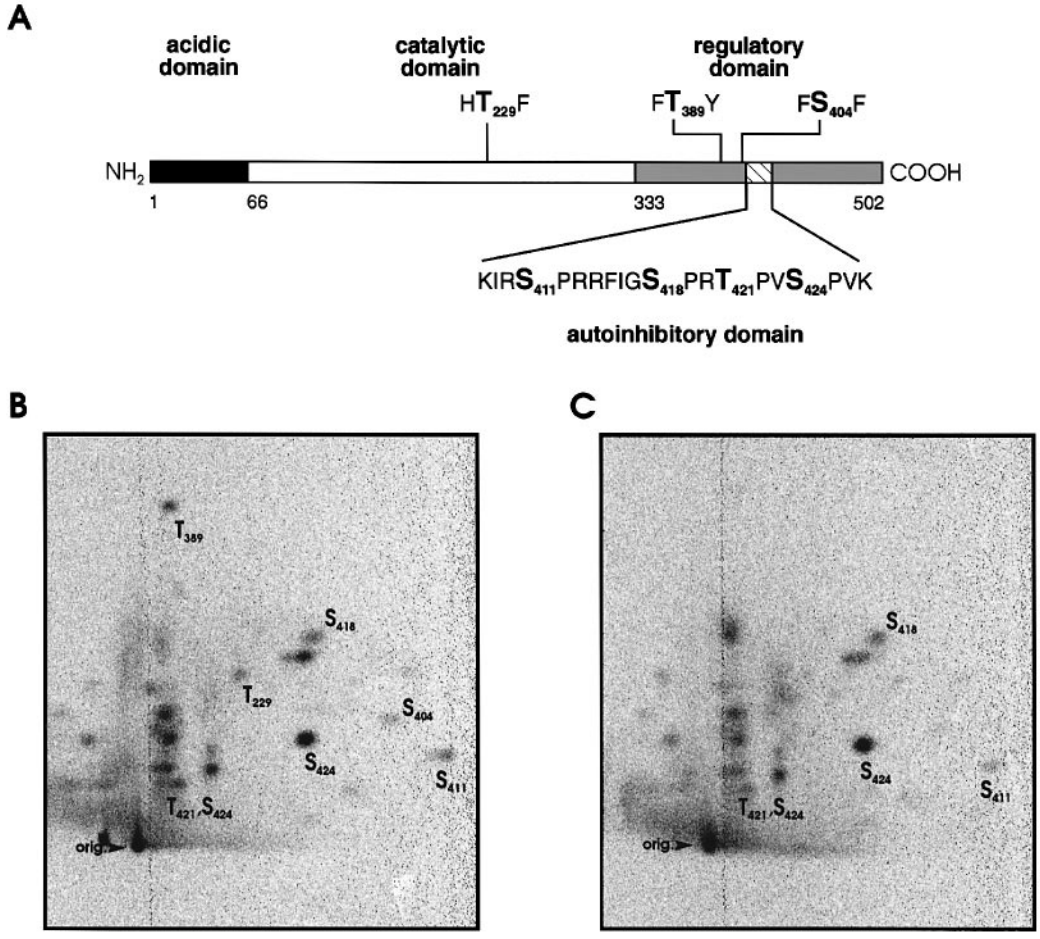

Fig. 3. Schematic representation of p70^{s6k} and effect of p70^{s6k}A229 on phosphorylation sites. (**A**) The principal rapamycin-sensitive phosphorylation sites are indicated above the model and the autoinhibitory domain phosphorylation sites are indicated below the model. The acidic amino-terminus, catalytic and regulatory domains are noted on the figure. (**B**) 293 cells were transfected with 1 μg of reporter myc-p70^{s6k}-GST alone or (**C**) together with 5 μg of myc-p70^{s6k}A229, serum starved for 24 h in phosphate-free DMEM and labelled with ^{32}P for 7 h. After 45 min stimulation with phosphate-free FCS (10%), the myc-p70^{s6k}-GST was precipitated and analysed on two-dimensional tryptic/chymotrypsin phosphopeptide maps employing PhosphoImager and ImageQuant software (Molecular Dynamics) as described in Materials and methods. The origin is marked with an arrow.

disome fraction than observed in NIH 3T3 cells (compare Figure 4C with Figure 1C, left panel). This difference may reflect the fact that it is more difficult to quiesce 293 cells, possibly as a consequence of being transformed with the E1A oncogene of adenovirus (Graham *et al.*, 1977). Following serum stimulation, most of the wild-type chimeric mRNA relocated from the monosome/disome fraction to polysomes of 7–8 ribosomes per transcript (Figure 4C). Similar results were obtained if the wild-type p70^{s6k} was co-transfected with the wild-type S16-hGH chimeric mRNA (data not shown). However, the serum-induced relocation of the wild-type chimeric mRNA was repressed in the presence of the dominant-interfering myc-p70^{s6k}A229 construct (compare Figure 4D with C), resembling the effect observed in serum-stimulated NIH 3T3 cells treated with rapamycin (Figure 1C, right panel). However, as noted for rapamycin, the dominant-interfering myc-p70^{s6k}A229 construct, despite abolishing p70^{s6k} activity, only partially suppresses the translational up-regulation of the S16-hGH chimeric mRNA. These findings support the hypothesis that serum-induced p70^{s6k} independent signalling pathways are also implicated in the translational up-regulation of 5′TOP mRNAs. Thus the dominant-interfering p70^{s6k}A229 mimics the inhibitory effects of rapamycin on the translation of 5′TOP mRNAs.

Rapamycin-resistant T389E mutant

A caveat with the dominant-interfering approach is that parallel pathways which bifurcate directly upstream of p70^{s6k} may also be inhibited. To address this issue, we asked whether a rapamycin-resistant mutant of p70^{s6k}, where the principal site of rapamycin-induced p70^{s6k} dephosphorylation, T389, has been changed to a glutamic acid (Pearson *et al.*, 1995; Dennis *et al.*, 1996), was sufficient to maintain serum-stimulated 5′TOP mRNA translation in the presence of the macrolide. As the transfection efficiency of 293 cells is high, the p70^{s6k}E389 construct should generate a signal above background, precluding the use of the wild-type reporter mRNA construct. Thus, the distribution of endogenous eEF-1α mRNA, which we have shown previously to be suppressed by rapamycin (Jefferies *et al.*, 1994a), was followed on polysome profiles from 293 cells transiently expressing

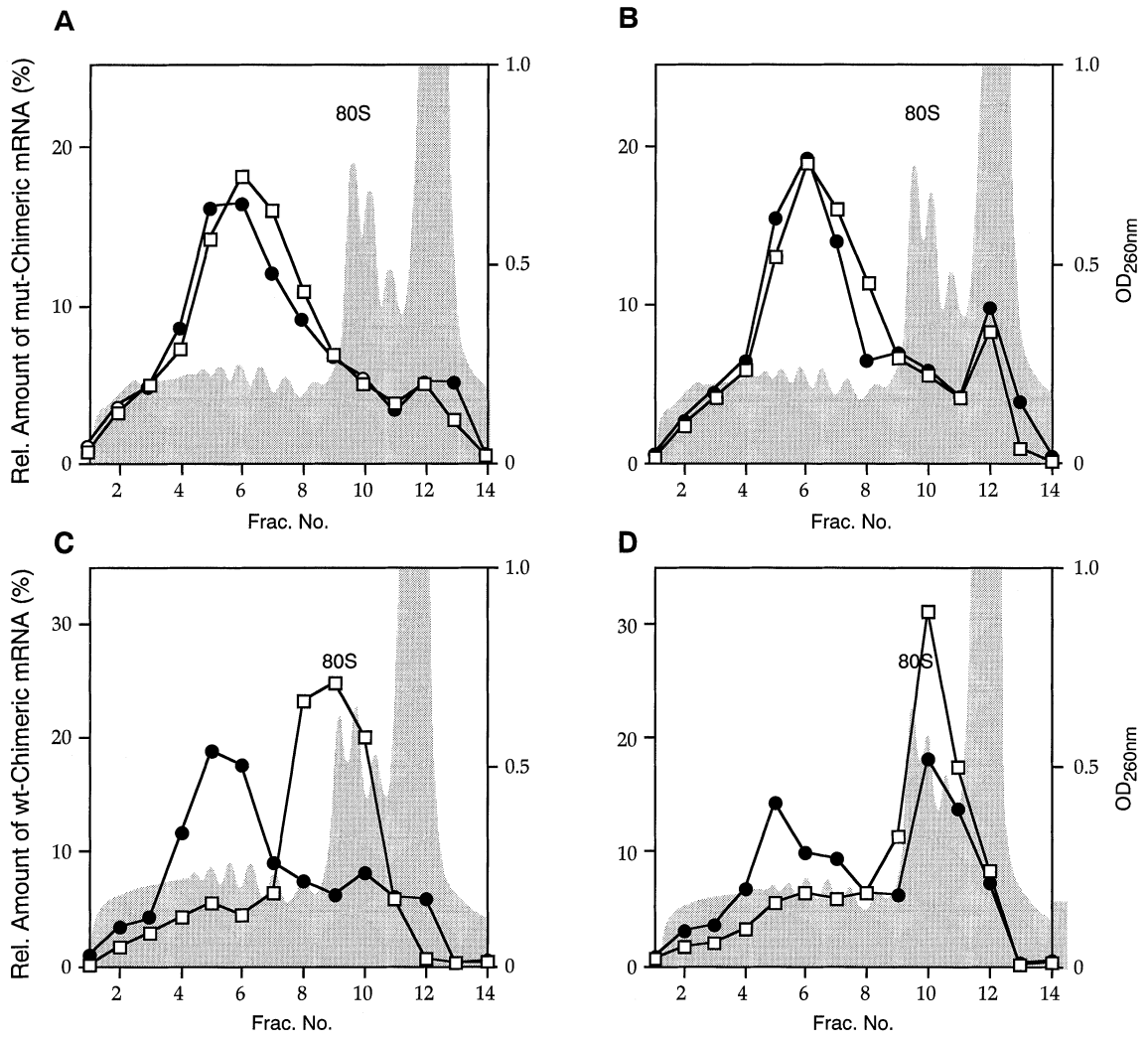

Fig. 4. Dominant-interfering effect of myc-p70^{s6k}A229 on the polysome distribution of chimeric mRNAs. 293 cells were transiently transfected with (**A**) 2 μg of cm5S16-hGH construct co-transfected with 10 μg of empty CMV-plasmid, (**B**) 2 μg of cm5S16-hGH construct co-transfected with 10 μg of myc-p70^{s6k}A229 plasmid, (**C**) 2 μg of wtS16-hGH construct co-transfected with 10 μg of empty CMV-plasmid and (**D**) 2 μg of wtS16-hGH construct co-transfected with 10 μg of myc-p70^{s6k}A229 plasmid. Cytoplasmic extracts were made from cells serum starved for 26 h (□) or serum starved and then stimulated with 10% FCS for 4 h (●). Extracts were analysed as described in Figure1 on 17.1–40% linear sucrose gradients. In each case, the shaded areas depict the $OD_{260\ nm}$ polysome gradient profile from the serum-stimulated 293 cell extract under the indicated conditions with the position of the 80S peak noted.

either the wild-type p70^{s6k} or the rapamycin-resistant p70^{s6k}E389. In quiescent cells, eEF-1α mRNA partitioned with monosome/disomes and mRNP particles in the presence of either wild-type myc-p70^{s6k} or the rapamycin-resistant mutant myc-p70^{s6k}E389 kinase construct (Figure 5A and C, respectively). As for the transiently expressed wild-type S16-hGH chimeric transcript (Figure 4C), more eEF-1α mRNA partitioned proportionally with monosome/disomes than observed earlier in 3T3 cells (Jefferies *et al.*, 1994a). Following serum stimulation, eEF-1α transcripts largely redistributed to polysomes of 11–12 ribosomes per transcript, with slightly higher levels recruited to polysomes in the case of cells expressing the myc-p70^{s6k}E389 construct (compare Figure 5B with D). We consistently have observed slightly higher levels of recruitment when employing either the p70^{s6k}E389 or the more active p70^{s6k}D3E–E389 variant. More importantly, as in the case of the stably expressed wild-type S16-hGH chimeric transcript, rapamycin treatment suppressed the translational up-regulation of the eEF-1α transcript (Figure 5B). In contrast, the rapamycin-resistant p70^{s6k}E389 construct almost completely protected eEF-1α from the inhibitory effects of rapamycin (Figure 5D). Indeed, if the efficiency of transfection is considered, which was between 70 and 80%, the protection would appear complete, in agreement with results obtained with the wild-type S16-hGH chimeric transcript (data not shown). Consistent with these findings, cells expressing p70^{s6k}E389, as compared with the wild-type construct, display high kinase activity following rapamycin treatment (Figure 5E). Although the p70^{s6k}E389 construct has high basal activity, its over-expression in the absence of mitogen stimulation does not induce the translational up-regulation of eEF-1α transcripts, indicating that p70^{s6k} activation is not sufficent to induce this response. Nevertheless, a rapamycin-resistant p70^{s6k} is sufficient in protecting 5′TOP mRNAs from the inhibitory effects of the macrolide and supports the hypothesis that the dominant-negative p70^{s6k}A229

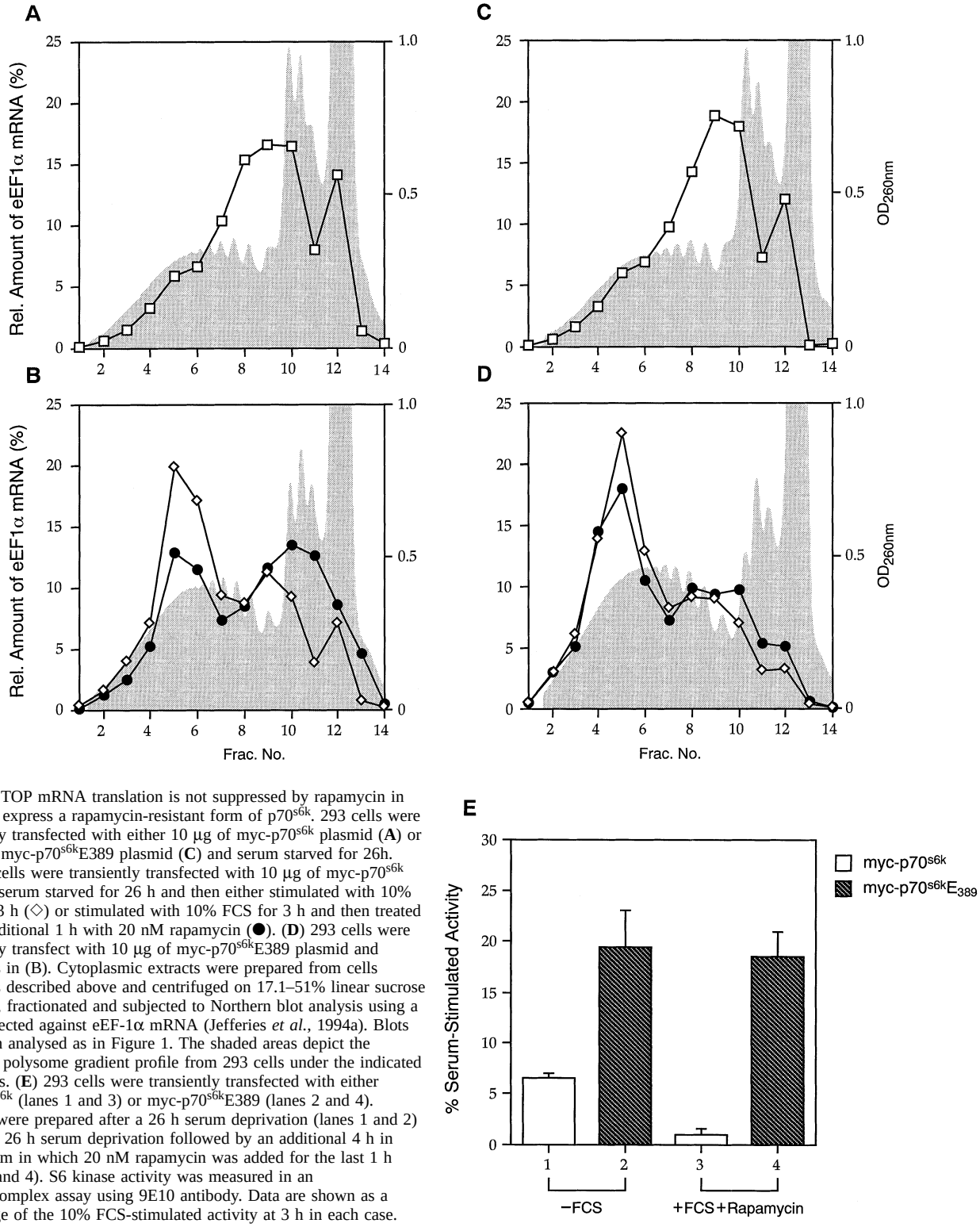

Fig. 5. 5′TOP mRNA translation is not suppressed by rapamycin in cells that express a rapamycin-resistant form of p70^{s6k}. 293 cells were transiently transfected with either 10 μg of myc-p70^{s6k} plasmid (**A**) or 10 μg of myc-p70^{s6k}E389 plasmid (**C**) and serum starved for 26h. (**B**) 293 cells were transiently transfected with 10 μg of myc-p70^{s6k} plasmid, serum starved for 26 h and then either stimulated with 10% FCS for 3 h (◇) or stimulated with 10% FCS for 3 h and then treated for an additional 1 h with 20 nM rapamycin (●). (**D**) 293 cells were transiently transfect with 10 μg of myc-p70^{s6k}E389 plasmid and treated as in (B). Cytoplasmic extracts were prepared from cells treated as described above and centrifuged on 17.1–51% linear sucrose gradients, fractionated and subjected to Northern blot analysis using a probe directed against eEF-1α mRNA (Jefferies *et al.*, 1994a). Blots were then analysed as in Figure 1. The shaded areas depict the $OD_{260\ nm}$ polysome gradient profile from 293 cells under the indicated conditions. (**E**) 293 cells were transiently transfected with either myc-p70^{s6k} (lanes 1 and 3) or myc-p70^{s6k}E389 (lanes 2 and 4). Extracts were prepared after a 26 h serum deprivation (lanes 1 and 2) or after a 26 h serum deprivation followed by an additional 4 h in 10% serum in which 20 nM rapamycin was added for the last 1 h (lanes 3 and 4). S6 kinase activity was measured in an immunocomplex assay using 9E10 antibody. Data are shown as a percentage of the 10% FCS-stimulated activity at 3 h in each case.

blocks translation of 5′TOP mRNAs through inhibition of endogenous p70^{s6k}.

Discussion

The data presented here point to a central role for p70^{s6k} in the selective translational up-regulation of 5′TOP mRNAs in response to mitogens and demonstrate that the inhibitory effects of rapamycin on the expression of this family of mRNAs is exerted through inhibition of the kinase. Furthermore, it is clear that the inhibitory effects of both rapamycin and the dominant-interfering p70^{s6k} mutant on 5′TOP mRNA translation require an intact polypyrimidine tract (Figures 1 and 4). This last finding is consistent with the fact that the dominant-interfering p70^{s6k} mutant preferentially blocks the equivalent phosphorylation sites in a reporter p70^{s6k} as does rapamycin (Figure 3 and Pearson *et al.*, 1995). A tentative model

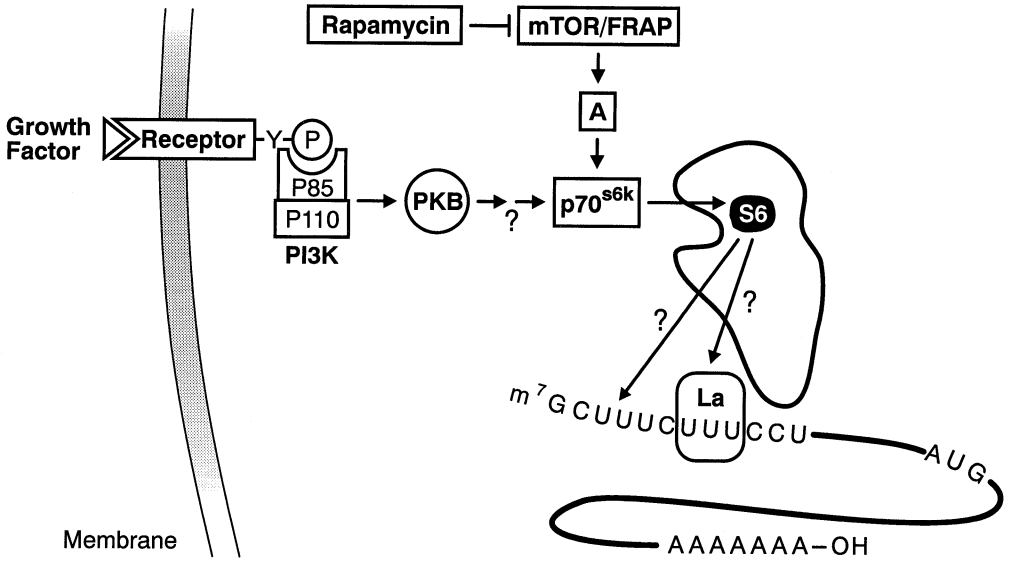

Fig. 6. A model of the p70^{s6k} signalling pathway leading to the translational up-regulation of 5′TOP mRNAs.

depicting the signalling pathway leading to the activation of the p70^{s6k}, and thus the translational up-regulation of 5′TOP mRNAs, has emerged recently (Brown and Schreiber, 1996) and is depicted in Figure 6. In brief, mitogen stimulation is speculated to first trigger the activation of the phosphatidylinositol 3-OH kinase followed by activation of protein kinase B which would function upstream of an as yet unidentified p70^{s6k} kinase (Downward, 1995). In contrast, mTOR/FRAP exerts its effects through a putative effector molecule which operates on the amino-terminus of p70^{s6k}, whose activity may or may not be regulated by mitogens (Weng *et al.*, 1995b; Dennis *et al.*, 1996). However, a number of recent observations are not wholly consistent with phosphatidylinositol 3-OH kinase and protein kinase B acting as upstream components of the p70^{s6k} signal transduction pathway (for a discussion, see Dennis *et al.*, 1996). Similarly, the existence of a rapamycin-sensitive effector molecule and the role of S6 in modulating the effects of p70^{s6k} on 5′TOP mRNAs have yet to be established (see below).

The mechanism by which the dominant-interfering p70^{s6k}A229 mutant blocks the same set of phosphorylation sites as rapamycin in the p70^{s6k} reporter is unknown; however, it is most likely through competing for a proximal upstream activator which is rapamycin sensitive. Indeed, recent studies have demonstrated that co-expression of either the wild-type kinase or a kinase-dead construct, in which the conserved lysine in the ATP-binding site has been mutated, also block the same set of sites as observed with the dominant-interfering p70^{s6k}A229 mutant (P.B.Dennis and G.Thomas, unpublished data). In all cases, an immediate upstream component is apparently sequestered, thus it is possible that parallel pathways that bifurcate upstream of p70^{s6k} also may be affected. In support of this model, recent studies have shown that insulin-induced 4E-BP1 phosphorylation is blocked by p70^{s6k}A229 (S.von Manteuffel, P.B.Dennis and G.Thomas, unpublished data), confirming earlier results that p70^{s6k} and 4E-BP1 lie on a common signalling pathway. However, the rapamycin-resistant p70^{s6k} mutant, while preventing rapamycin from inhibiting the translation of 5′TOP mRNAs (Figure 5), does not protect 4E-BP1 from dephosphorylation (S.von Manteuffel, P.B.Dennis and G.Thomas, unpublished data), suggesting that 4E-BP1 may not be significantly implicated in the regulation of these mRNAs. This observation is consistent with the finding that overexpression of eIF-4E, the inhibitory target of 4E-BP1, does not result in the selective up-regulation of 5′TOP mRNAs (Avni *et al.*, 1994), and that the mutant cm5S16-hGH chimeric mRNA, containing the disrupted polypyrimidine tract, localizes to polysomes in the absence of mitogenic stimulation (Figures 1 and 3). Moreover, given the role of unphosphorylated 4E-BP1 in inhibiting general eIF-4E cap-dependent translation (Pause *et al.*, 1994), it is difficult to reconcile that rapamycin blocks 4E-BP1 phosphorylation but has little effect on global protein synthesis in Swiss mouse 3T3 cells (Jefferies *et al.*, 1994a). It has been suggested that eIF-4E may be primarily important in the translation of a subset of mRNAs which contain a highly structured 5′UTR, such as ornithine decarboxylase (Shantz and Pegg, 1994), ornithine aminotransferase (Fagan *et al.*, 1991), cyclin D_1 (Rosenwald *et al.*, 1995), Q23 or p23 (Bommer *et al.*, 1994) and myc (DeBenedetti *et al.*, 1994). Nevertheless, we have noted that the translation of ornithine decarboxylase mRNA, which can be augmented by the overexpression of eIF-4E (Shantz and Pegg, 1994), is unaffected by treating either Swiss 3T3 or 293 cells with rapamycin (data not shown). It may be that these differences can be attributed to cell type specificity or that additional factors are involved which have yet to be described. Indeed, there are a number of eIF-4E-binding protein homologues (Pause *et al.*, 1994), thus 4E-BP1′s role in regulating translation may be more subtle.

The requirement for p70^{s6k} activity for full translational up-regulation of 5′TOP mRNAs implicates a downstream target of the protein synthetic apparatus as being responsible for mediating this response. Despite intensive efforts, the only relevant *in vivo* physiological substrate found to date for p70^{s6k} is 40S ribosomal protein S6 (Ferrari and Thomas, 1994). In response to mitogenic signals, S6 becomes increasingly phosphorylated on five of seven

serines residing at the carboxy-terminus of the protein, including S235, S236, S240, S244 and S247 (Krieg *et al.*, 1988). Moreover, phosphate release studies have shown that phosphorylation proceeds in an ordered fashion *in vivo* and *in vitro*: S236→S235 or S240→S244→S247 (Bandi *et al.*, 1993). Employing a number of approaches, S6 has been localized to the small head region of the 40S ribosomal subunit, where it has been cross-linked to a number of translational components residing in or at the tRNA/mRNA-binding site, including the 3′ end of 18S rRNA, tRNA, initiation factors and mRNA (Nygard and Nilsson, 1990). In addition, S6 is one of the few proteins of the 40S ribosomal subunit that has been cross-linked directly to the 28S rRNA component of the 60S subunit (Nygard and Nilsson, 1990), suggesting that it may lie at the interface between the two ribosomal subunits. The possibility that S6 may interact with mRNA and/or initiation factors is of potential functional significance. Nonetheless, mutation of the serines equivalent to S235 and S236 in the *Saccharomyces cerevisiae* homologue of S6, which lacks the last three sites of phosphorylation, has little effect on global translation, vegetative growth or the pattern of translation (Johnson and Warner, 1987). However, *S.cerevisiae* ribosomal proteins and elongation factors do not contain 5′TOPs and, unlike mammals, *S.cerevisiae* appears to acutely regulate the production of protein synthetic machinery at the transcriptional level. Furthermore, it is clear in mammalian cells that the p70^{s6k} signalling pathway is not the only signalling pathway leading to the selective translational up-regulation of 5′TOP mRNAs, as neither rapamycin nor the dominant-interfering p70^{s6k}A229 mutant completely ablate the translational up-regulation of 5′TOP mRNAs (Figures 1 and 3, Jefferies *et al.*, 1994a). It may be that in *S.cerevisiae* the functional importance of S6 phosphorylation is masked by a redundant signalling pathway. Certainly, many functional pathways in *S.cerevisiae* are redundant and require advanced genetic approaches to uncover their importance, such as the employment of synthetic lethal screens (see Posas *et al.*, 1996). In support of increased S6 phosphorylation playing a critical role in translational up-regulation of 5′TOP mRNAs, earlier studies demonstrated that 40S ribosomal subunits, containing predominantly the highly phosphorylated forms of S6, bound poly(U) more strongly than unphosphorylated ribosomes and that poly(U) dissociated from the phosphorylated ribosomes much more slowly than from their unphosphorylated counterparts (Gressner and van de Leur, 1980). As the 5′TOP is mostly made up of Us, it might be speculated that phosphorylated forms of S6 interact directly with polypyrimidine tract-containing mRNAs, increasing the possibility of forming a stable initiation complex (see Figure 6).

In elucidating the signalling pathway leading to the translational up-regulation of 5′TOP mRNAs, it should be noted that, in *Xenopus* and mouse cells, a protein of M_r 57 kDa has been cross-linked specifically to the polypyrimidine tract of ribosomal protein mRNAs L4 and L32 (Kaspar *et al.*, 1992; Cardinali *et al.*, 1993). Recent studies have identified this protein as the La autoantigen (Pellizzoni *et al.*, 1996), which elicits autoantibody production in patients with systemic lupus erythematosus and Sjögren's syndrome. La was first implicated in RNA polymerase III transcription termination (Gottlieb and Steitz, 1989) and, more recently, in the replication of Sindbis virus (Pardigon and Strauss, 1996), as well as stimulating the translation of viral and cap-dependent mRNAs (Svitkin *et al.*, 1994a,b, 1996). La possess an inherent ATPase activity (Bachmann *et al.*, 1990) and an RNA-binding motif (Chambers *et al.*, 1988), and binds to poly(U) with high avidity (Topfer *et al.*, 1993). La derived from extracts of either resting or serum-stimulated cells binds with the same ability to the 5′TOP as measured by mobility shift assays employing an oligonucleotide identical in sequence to the first 34 bases of ribosomal protein L32 (Kaspar *et al.*, 1992). Furthermore, binding is cap independent and is competed away effectively by an oligonucleotide corresponding to the polypyrimidine tract or poly(U) (Kaspar *et al.*, 1992). Mutations in the polypyrimidine tract, which ablate the translational control of 5′TOP mRNAs, also impair the binding of La (Pellizzoni *et al.*, 1996). To date, no direct relationship *in vivo* has been demonstrated between the binding of La to the polypyrimidine tract and the translational up-regulation of 5′TOP mRNAs. Given La's ability to bind to 5′TOP mRNA polypyrimidine tracts independently of growth state and, in parallel, to facilitate cap-dependent translation, it is difficult to conceive a functional role for La as a selective repressor of 5′TOP mRNA translation. However, a model could be envisaged whereby a second translational factor, such as phosphorylated S6, could bind to La in a specific context, such as the polypyrimidine tract, leading to the selective translation of 5′TOP mRNAs (Figure 6).

Consistent with the role of p70^{s6k} in regulating 5′TOP-containing ribosomal protein mRNAs, we have found recently that a P element-induced mutation in the *Drosophila* homologue (M.Stewart, F.Zilberman, C.Berry, S.Kozma, and G.Thomas, unpublished data) of the p70^{s6k} gene (Stewart *et al.*, 1996) exhibits a phenotype markedly resembling that of the *Minute*, *bobbed* and *mini* mutants, which affect the ribosomal protein and rRNA genes of *Drosophila* (see Andersson *et al.*, 1996). Interestingly, rapamycin also has been shown to inhibit rRNA synthesis (Mahajan, 1994) as well as cyclin D_1–Cdk4 and cyclin E–Cdk2 activation (Kato *et al.*, 1994; Nourse *et al.*, 1994), which through hyper-phosphorylation of the retinoblastoma protein have been implicated in the regulation of both RNA polymerase I and III (Cavanaugh *et al.*, 1995; White *et al.*, 1996), responsible for regulating rRNA synthesis. The inhibitory effects of rapamycin on cyclin D_1–Cdk4 and cyclin E–Cdk2 are thought to be mediated by the p27 inhibitor (Kato *et al.*, 1994; Nourse *et al.*, 1994); however, cells derived from p27 knockout animals have been shown to be either completely (Nakayama *et al.*, 1996) or partially rapamycin sensitive for growth (Luo *et al.*, 1996), consistent with data which would suggest an alternative target (Kato *et al.*, 1994). At this point, it will be important to establish whether the effects of rapamycin on cyclin D_1–Cdk4 and cyclin E–Cdk2 activation and rRNA synthesis are mediated in part through p70^{s6k}. Employing the p70^{s6k} constructs described here should make it possible to resolve these issues.

Materials and methods

Cell culture, cytoplasmic polysome extraction and sucrose gradients

NIH 3T3 cell lines were seeded and maintained on 15 cm tissue culture plates in standard Dulbecco's modified Eagles medium (DMEM)

containing 10% fetal calf serum (FCS) and 100 μg/ml of G418 (geneticin) for maintaining the stable transfectants. NIH 3T3 cells were grown to ~80% confluence, the medium was replaced by DMEM containing 0.5% FCS and the cells cultured for 40 h. The medium was then replaced by DMEM containing 0.1% bovine serum albumin (BSA) and the cells cultured for a further 1 h before treating the cells as described in the figure legends. Rapamycin (2 mM in ethanol) was diluted 1:100 in medium before being added to the cells at a final concentration of 20 nM. Cytoplasmic extracts were prepared as described for Swiss 3T3 cells (Jefferies *et al.*, 1994b). Human kidney 293 cells were maintained on 10 cm tissue culture plates in DMEM containing 10% FCS. Human 293 cells were seeded at 1×10^6 cells per 10 cm plate and cultured for 24 h. They were then transfected by a modified calcium phosphate method (Dennis *et al.*, 1996). After 12–14 h, the cells were washed three times then cultured in DMEM without serum for 24–26 h. For extracts, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed directly on the plate by the addition of 300 μl of hypotonic lysis buffer containing 1.5 mM KCl, 2.5 mM $MgCl_2$, 5 mM Tris–HCl pH 7.4, 1% Triton X-100 and 1% deoxycholate. The lysate was collected and treated as for Swiss 3T3 cells (Jefferies *et al.*, 1994b). Briefly, 300 μl of cytoplasmic extract were applied to linear sucrose gradients containing 80 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris–HCl pH 7.4, and 1 mM dithithreitol, which were centrifuged in an SW41 Beckmann rotor at 36 000 r.p.m. for 2 h at 4°C. Fractionation of the sucrose gradients, isolation of RNA, Northern blot analysis and 5′ end labelling of the oligonucleotide probes were all carried out as described earlier (Jefferies *et al.*, 1994b).

Oligonucleotide probes

The oligonucleotide hGH probe is complementary to 57 bases of exon 2 of the human growth hormone gene 753–697 (GGT AGG TGT CAA AGG CCA GCT GGT GCA GAC GAT GGG CGCGGA GAC TAG CGT TGT CAA). The eEF-1α oligonucleotide probe is complementary to the first 57 coding bases of human eEF-1α (DDBJ/EMBL/GenBank accession No. J04616/J04617).

Transient transfections

Calcium phosphate transfection of plasmid DNA into 293 cells was performed as described in Okayama and Chen (1988). One ml of transfection mix containing the amounts of DNA stated in the figure legends was applied to the 293 cells on 10 cm culture plates. Particular attention was paid to the pH of the BBS [25 mM *N*-, *N*-bis(2-hydroxy-ethyl)-2-amino-ethanesulfonic acid] solution and to the precipitation times to achieve optimal DNA–calcium phosphate precipitation and transfection efficiency (Jordan *et al.*, 1996). The efficiency of transfection was assessed by transiently transfecting an RSV-lacZ plasmid into 293 cells under conditions indentical to those for $p70^{s6k}$ constructs and then performing β-galactosidase histochemical staining of the cells as described previously (Sanes *et al.*, 1986).

Western blot analysis, kinase assays and two-dimensional tryptic phosphopeptide analysis

Cells used for Western blot, kinase assays and two-dimensional tryptic phosphopeptide analysis were extracted in buffer A as described (Ferrari *et al.*, 1993; Dennis *et al.*, 1996). Western blotting and immune staining of expressed proteins were performed as described (Dennis *et al.*, 1996). Both 9E10 and 12CA5 are monoclonal antibodies against the myc and HA epitope tags, respectively. Visualization and quantitation of specific proteins were carried out using fluorescein isothiocyanate (FITC)-labelled secondary antibodies (Dako) followed by fluorometry and analysis with Imagequant software (Molecular Dynamics). For S6 kinase assays of myc-$p70^{s6k}$-GST, 20 μg of protein cell extract were precipitated with glutathione beads (Sigma) for 30 min at 4°C. The beads were washed and assayed as for protein A–Sepharose CL-4B (Pharmacia) as described (Lane *et al.*, 1992). 12CA5 antibody was used to immunoprecipitate HA-$p44^{mapk}$ from 20 μg of protein cell extract and assayed for MBP kinase activity (de Vries-Smits *et al.*, 1992). The immunocomplex assays of endogenous $p70^{s6k}$ activity from 20 μg of protein were performed utilizing the M5 polyclonal antibody as previously described (Lane *et al.*, 1992). 9E10 monoclonal antibody was used in immunocomplex assays for myc-$p70^{s6k}$ and myc-$p70^{s6k}$E389 transiently expressed in 293 cells (Pearson *et al.*, 1995). Two-dimensional peptide mapping of ^{32}P-labelled peptides was carried out as previously described (Dennis *et al.*, 1996).

Recombinant plasmids

Construction of the wild-type S16-hGH construct and the mutant cm5S16-hGH construct is detailed in Levy *et al.* (1991). All $p70^{s6k}$ constructs used were tagged by the insertion of the myc 9E10 epitope immediately following the $p70^{s6k}$ isoform initiator ATG codon as previously described (Ming *et al.*, 1994). Site-directed mutagenesis was performed using the Altered site II Mutagenesis System (Promega) according to the manufacturer's instructions. All $p70^{s6k}$ constructs were placed in a cytomegalovirus-driven expression plasmid (CMV-plasmid) and confirmed by DNA sequencing. Creation of the myc-$p70^{s6k}$A229 and the myc-$p70^{s6k}$E389 construct are described in Pearson *et al.* (1995). HA-$p44^{mapk}$ construct was a gift from J.Pouysségur (Meloche *et al.*, 1992). The construction of the $p70^{s6k}$-GST is to be described elsewhere.

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