Molecular and Cellular Biology, Sept. 1997, p. 5426–5436 Vol. 17, No. 9
0270-7306/97/$04.00+0
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The Insulin-Induced Signalling Pathway Leading to S6 and Initiation Factor 4E Binding Protein 1 Phosphorylation Bifurcates at a Rapamycin-Sensitive Point Immediately Upstream of $p70^{s6k}$

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Received 18 March 1997/Returned for modification 17 April 1997/Accepted 30 May 1997

Employing specific inhibitors and docking-site mutants of growth factor receptors, recent studies have indicated that the insulin-induced increase in 40S ribosomal protein S6 and initiation factor 4E binding protein 1 (4E-BP1) phosphorylation is mediated by the mTOR/FRAP-$p70^{s6k}$ signal transduction pathway. However, it has not been resolved whether the phosphorylation of both proteins is mediated by $p70^{s6k}$ or whether they reside on parallel pathways which bifurcate upstream of $p70^{s6k}$. Here we have used either rapamycin-resistant, kinase-dead, or wild-type $p70^{s6k}$ variants to distinguish between these possibilities. The rapamycin-resistant $p70^{s6k}$, which has high constitutive activity, was able to signal to S6 in the absence of insulin and to prevent the rapamycin-induced block of S6 phosphorylation. This same construct did not increase the basal state of 4E-BP1 phosphorylation or protect it from the rapamycin-induced block in phosphorylation. Unexpectedly, the rapamycin-resistant $p70^{s6k}$ inhibited insulin-induced 4E-BP1 phosphorylation in a dose-dependent manner. This effect was mimicked by the kinase-dead and wild-type $p70^{s6k}$ constructs, which also blocked insulin-induced dissociation of 4E-BP1 from initiation factor 4E. Both the kinase-dead and wild-type constructs also blocked reporter $p70^{s6k}$ activation, although only the kinase-dead $p70^{s6k}$ had a dominant-interfering effect on S6 phosphorylation. Analysis of phosphopeptides from reporter 4E-BP1 and $p70^{s6k}$ revealed that the kinase-dead $p70^{s6k}$ affected the same subset of sites as rapamycin in both proteins. The results demonstrate, for the first time, that activated $p70^{s6k}$ mediates increased S6 phosphorylation in vivo. Furthermore, they show that increased 4E-BP1 phosphorylation is controlled by a parallel signalling pathway that bifurcates immediately upstream of $p70^{s6k}$, with the two pathways sharing a common rapamycin-sensitive activator.

In insulin-responsive cells, hormonal stimulation provokes the coordinate activation of a complex network of signalling pathways which are involved in the regulation of specific metabolic processes (71). Critical among these affected metabolic processes is the activation and maintenance of high rates of protein synthesis, leading to both global and selective changes in the pattern of translation (14, 23, 56). Recent studies have suggested that in insulin-sensitive tissues, such as liver, heart, skeletal muscle, and adipose tissue, the increase in protein synthesis is triggered by ligand-induced activation of the receptor and propagated intracellularly through the phosphorylation of the insulin receptor substrate IRS-1 (66). IRS-1 is thought to mediate the insulin signal by inducing the activation of distinct kinases which then target specific components of the translational apparatus (45). In several cases, there is considerable knowledge concerning the functional roles of specific translational components in protein synthesis and of the effect of phosphorylation on their individual activities (48, 65). Much less is known regarding the identities of the kinases responsible for modulating the phosphorylation states of specific translational components or of the signalling pathways which regulate their activities. A number of recent studies have suggested that $p70^{s6k}$ may be intimately involved in mediating the effect of hormones on global as well as selective patterns of translation (2, 27, 45), through the multiple phosphorylation of the 40S ribosomal protein S6 (20) and possibly the recently identified initiation factor 4E (eIF4E) binding protein, 4E-BP1 (53) or PHAS-I (40).

Mitogen-induced phosphorylation and activation of $p70^{s6k}$ appear to play an important role during the G_1 phase of the cell cycle (10, 35, 37, 55, 58), consistent with the putative function of $p70^{s6k}$ in regulating increased S6 and 4E-BP1 phosphorylation (2, 45, 68). In parallel it has been demonstrated that the immunosuppressant rapamycin, a bacterial macrolide, negates mitogen-induced activation of $p70^{s6k}$ by preventing the acute phosphorylation of a specific subset of sites, including T_{229}, T_{389}, S_{404}, and S_{411} (54). These sites were found to be flanked by large aromatic residues, with the exception of S_{411}, which exhibits an S/TP motif, as do the remaining three unaffected phosphorylation sites (18, 54). Of the rapamycin-sensitive sites, the principal site of rapamycin-induced dephosphorylation leading to $p70^{s6k}$ inactivation was identified as T_{389}, which resides in the linker region, coupling the catalytic and autoinhibitory domains (54). Conversion of this site to an acidic amino acid confers high basal activity and rapamycin resistance on the kinase (54). The mechanism by which rapamycin brings about $p70^{s6k}$ inactivation is not direct. The macrolide, in a gain-of-function inhibitory complex with the immunophilin FKBP12, binds to a high-molecular-weight protein termed mTOR or FRAP (6, 60). Based on structural similarities, mTOR/FRAP is thought to function as either a lipid or protein kinase (26). A mutant form which is unable to bind the

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FKBP12-rapamycin inhibitory complex has been shown to protect p70^{s6k} from inactivation by the macrolide, indicating that mTOR/FRAP is an upstream component of the p70^{s6k} signalling pathway (7). Interestingly, the rapamycin-FKBP12 gain-of-function inhibitory complex does not appear to interfere with any of the kinases which modulate p70^{s6k} function but instead appears to act through an unidentified effector molecule operating through the amino terminus of the kinase (13, 69). Although rapamycin treatment in some cell types is known to inhibit the activation of specific cyclin-dependent kinases at the G_1/S boundary (31, 50), it potently and selectively blocks p70^{s6k} activation throughout the G_1 phase of the cell cycle in all cell types examined (8).

Rapamycin-induced p70^{s6k} dephosphorylation and inactivation have been shown to be paralleled by dephosphorylation of S6 (10, 28) and 4E-BP1 (4, 68). S6 is an integral 40S ribosomal protein which resides near or at the mRNA binding site, on that surface of the subunit which interacts directly with the larger 60S ribosomal subunit (51). Mitogen stimulation of cells to proliferate leads to multiple, ordered phosphorylation of S6 at five serines which are located at the carboxy terminus of the protein (3, 33). In vitro, p70^{s6k} is capable of phosphorylating all of the same sites as observed in vivo (17), although corroborative in vivo data are lacking. The increase in S6 phosphorylation is hypothesized to be involved in the rapid and selective translational upregulation of a family of mRNAs which contain an oligopyrimidine tract at their 5′ transcriptional start sites (27, 28, 28a). As with S6, it has recently been shown that 4E-BP1 is also phosphorylated when cells are stimulated to proliferate (53), although, with the exception of S_{64} (25), little is known concerning the sites of phosphorylation. Increased phosphorylation of 4E-BP1 leads to its release from eIF4E, allowing the initiation factor to then interact with the eIF4G subunit of the mRNA cap-binding protein complex (42). The rapamycin-induced block in 4E-BP1 phosphorylation is followed more slowly by a partial suppression of cap-dependent mRNA translation (4). Consistent with this finding, employment of pharmacological agents which indirectly lead to the selective inhibition of p70^{s6k} activation, as well as docking-site mutants of IRS-1 (45, 68) and the platelet-derived growth factor receptor (68), has revealed that increased 4E-BP1 phosphorylation is mediated by the same signalling pathway as that which modulates p70^{s6k} and S6 phosphorylation. These findings and others have led to the hypothesis that 4E-BP1 phosphorylation may be mediated by p70^{s6k} (2, 45). The possibility that 4E-BP1 is a direct target seems unlikely, because 4E-BP1 neither serves as an in vitro substrate for p70^{s6k} (25) nor contains any p70^{s6k} recognition motifs (21) or phosphorylation sites similar to those described for S6 (34). However, these observations do not distinguish between the possibilities of p70^{s6k} (i) acting as an upstream modulator of a 4E-BP1 kinase or (ii) instead bifurcating at some point downstream of mTOR/FRAP on a signalling pathway that is parallel but does not lead to increased 4E-BP1 phosphorylation.

In establishing the relative importance of S6 and 4E-BP1 phosphorylation in mediating the effects of insulin-induced p70^{s6k} activation on protein synthesis, it is necessary to resolve whether both proteins are downstream targets of the kinase. To address this question, advantage was taken of a number of p70^{s6k} variants, including the wild-type enzyme as well as two mutants which display either a rapamycin-resistant or a dominant-interfering phenotype. These constructs, in combination with activity assays and phosphopeptide analysis, were used to define the role of p70^{s6k} in mediating S6 and 4E-BP1 phosphorylation as well as the point in the mTOR/FRAP-p70^{s6k} signalling pathway at which these two events diverge from one another. The impact of these findings is discussed in relation to the role of S6 and 4E-BP1 phosphorylation in global and general translation.

MATERIALS AND METHODS

Cell culture, transfection, and extract preparation. Human embryonic kidney 293 cells (ATCC CRL 1573) were maintained as previously described (19). For transient expression of cDNA, 10^6 cells per 10-cm-diameter dish were seeded in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum 24 h prior to transfection, which was performed by a modified calcium phosphate precipitation procedure (52). After 12 h, cells were washed twice with DMEM and then incubated for an additional 24 h in DMEM prior to stimulation with 1 μM insulin (Sigma) in the absence or presence of 20 nM rapamycin. Cell extracts for Western blot analysis, affinity binding to 7-methyl-GTP–Sepharose (m^{7}GTP-Sepharose), and kinase assays were prepared as described earlier (68).

Metabolic labelling. Transiently transfected 293 cells were washed in DMEM lacking phosphate and were starved for 24 h. After incubation for 1 h in the presence of 0.2 mCi of $^{32}P_i$ (Amersham) per ml, cells were or were not treated with 20 nM rapamycin for 15 min prior to treatment with 1 μM insulin for 30 min. For preparation of ribosomal proteins, cells were washed twice with 5 ml of ice-cold phosphate-buffered saline, drained, and scraped into 1 ml of hypotonic buffer containing 1% Triton X-100 and 1% deoxycholate (64). Nuclei were removed by low-speed centrifugation for 5 min at 6,000 rpm in an Eppendorf tabletop centrifuge. The supernatant was first underlaid with 0.6 ml of 0.5 M sucrose-containing buffer A followed by 0.6 ml of 1.5 M sucrose-containing buffer B (64). Total ribosomes were pelleted by centrifugation at 2°C for 17 h at 75,000 rpm in a TLA 100.3 rotor (Beckman). Ribosomal proteins were prepared as described earlier (64), except that after acetone precipitation, the protein pellet was dissolved in 50 μl of 1× sodium dodecyl sulfate (SDS) sample buffer and boiled for 10 min (24). Equal amounts of each sample were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on Immobilon-P membranes (Millipore). Equal loading of ribosomal proteins was determined by employing an antibody specific for 60S ribosomal protein L7a (72), and $^{32}P_i$ incorporation into S6 was detected with a PhosphorImager (Molecular Dynamics) and analyzed with ImageQuant software (Molecular Dynamics).

Immunoprecipitation, kinase assay, and Western blotting. For in vitro kinase assays of myc–p70^{s6k}–glutathione *S*-transferase (GST), cell extracts (25 μg of total protein) were precipitated with glutathione-Sepharose (Sigma) for 30 min at 4°C. The beads were washed and assayed as previously described for kinase activity (38). For myc-p70^{s6k} assays, extracts were incubated with the anti-myc 9E10 monoclonal antibody (32), and kinase assays were performed as previously described (39). HA-p44^{mapk} assays were performed by incubating 20 μg of total protein with the antihemagglutinin (anti-HA) 12CA5 monoclonal antibody (32), with kinase activity measured against myelin basic protein (15). Western blot analysis of 4E-BP1 was carried out as previously described (68), except that the 12CA5 antibody was used for detection and the bisacrylamide content of the gel was lowered to 0.25%. For quantification of expressed protein from transfected cDNAs, 25 μg of total cell protein was analyzed by SDS-PAGE, the proteins were electrophoretically transferred to Immobilon-P membranes, and Western blot analysis was performed with either the anti-myc 9E10 or the anti-HA 12CA5 as the primary antibody. A tertiary fluorescein isothiocyanate-coupled antibody (DAKO) was used for visualization of Western blots with a scanning fluorimeter (Molecular Dynamics) and analysis with ImageQuant software. Relative units obtained with the PhosphorImager for kinase activity were normalized for expressed transfected kinase as quantified by fluorimetry with ImageQuant software.

Two-dimensional phosphopeptide mapping and stoichiometry measurements. Transiently transfected 293 cells were labelled with $^{32}P_i$ (0.2 mCi/ml) 6 h prior to extraction. myc-p70^{s6k}-GST was precipitated from labelled extracts with glutathione-Sepharose (Sigma), whereas the HA-4E-BP1 construct was immunoprecipitated with the 12CA5 antibody and protein G-Sepharose (Pharmacia). The precipitated proteins were further purified by SDS-PAGE and electroeluted from the gel. Trichloroacetic acid precipitation, performic acid oxidation, and trypsin-chymotrypsin digestion were carried out as described previously (13) except that the HA-4E-BP1 protein was precipitated with 25% trichloroacetic acid following electroelution. Two-dimensional phosphopeptide mapping was performed as described previously (13), and labelled phosphopeptides were visualized with a PhosphorImager. Relative stoichiometry measurements of HA-4E-BP1 involved resolution of the electroeluted ^{32}P-labelled material by SDS-PAGE followed by Western blotting. HA-4E-BP1 levels were determined by using a 12CA5 primary antibody and a fluorescein isothiocyanate-conjugated tertiary antibody. The stained proteins were visualized by scanning fluorimetry, whereas relative ^{32}P incorporation was measured with the PhosphorImager. Stoichiometry values were determined as the ratio of ^{32}P incorporation to the respective levels of HA-4E-BP1.

m^{7}GTP-Sepharose affinity assay. Cell extracts from transfected 293 cells corresponding to 150 μg of total protein were incubated with 30 μl of m^{7}GTP-Sepharose 4B (Pharmacia) in a total volume of 250 μl of LCB buffer (20 mM Tris HCl [pH 7.4], 0.2 mM EDTA, 100 mM KCl, 1 mM phenylmethylsulfonyl fluoride, and 7 mM β-mercaptoethanol). After 1 h of incubation at 4°C, unbound

proteins were removed by three 1-ml washes with LCB. Bound proteins were released by boiling in 20 μl of 1× SDS sample buffer (24) and analyzed by Western blotting with the anti-4E-BP1 antibody 11208 (22) or the anti-eIF4E antibody 10C6 (22) and a swine anti-rabbit horseradish peroxidase-coupled secondary antibody (DAKO). The horseradish peroxidase signal was detected with Super Signal TM Substrate (Pierce) and visualized with Biomax film (Kodak). Due to the multiple HA tags, the ectopically expressed HA-4E-BP1 migrates at a much higher molecular weight than endogenous 4E-BP1, which under the gel conditions employed migrates near the dye front of the gel.

Recombinant cDNA. Generation of the myc-$p70^{s6k}D_3E\text{-}E_{389}$ mutant (54), myc-$p70^{s6k}$ (47), and HA-$p44^{mapk}$ (44) has been described elsewhere. For HA-4E-BP1 the coding sequence of human 4E-BP1 was inserted in frame in the vector pACTAG-2 (a kind gift from M. Tremblay and A. Charest, McGill University), which puts three HA tags at the N terminus of 4E-BP1. By using the pAlter mutagenesis system (Promega), the 3′ stop codon of the full-length N-terminally myc-tagged $p70^{s6k}$ cDNA clone was replaced with a sequence which inserted a consensus for a thrombin cleavage site and 3′ *Pst*I and *Not*I sites. GST was amplified from pGEX-2T (Pharmacia) by PCR with *Pfu* polymerase (Stratagene) and oligonucleotides which introduced a 5′ *Pst*I site (GACTGCAGT CCCCTATACTAGGTTAT) and a 3′ *Not*I site (ATGCGGCCGCTCAATCCG ATTTTGG). The GST PCR product was digested with *Pst*I and *Not*I and subcloned into cognate sites in a cytomegalovirus (CMV)-driven plasmid. $p70^{s6k}$ was cloned into *Xba*I/*Pst*I sites in frame with the GST to generate the final myc-$p70^{s6k}$-GST construct.

RESULTS

A rapamycin-resistant $p70^{s6k}$ mutant protects S6 from dephosphorylation. To assess whether an insulin-induced rapamycin-sensitive phosphorylation event, such as the phosphorylation of 4E-BP1, is mediated by $p70^{s6k}$, it is essential to have a mutant which is resistant to the macrolide but unimpaired in its ability to signal downstream. In the $p70^{s6k}D_3E\text{-}E_{389}$ mutant, T_{389}, the principal target of rapamycin-induced $p70^{s6k}$ inactivation (13, 54), has been replaced by a glutamic acid, and the four S/TP phosphorylation sites in the autoinhibitory domain have been changed to either aspartic or glutamic acid (13). Compared with the wild-type $p70^{s6k}$, in transient-transfection assays, the $p70^{s6k}D_3E\text{-}E_{389}$ mutant exhibits high basal kinase activity (Fig. 1A, upper and middle panels, compare lanes 1 and 4), which is stimulated by the addition of insulin (Fig. 1A, upper and middle panels, compare lanes 2 and 5). More importantly, compared with the wild-type $p70^{s6k}$, the $p70^{s6k}D_3E\text{-}E_{389}$ mutant is largely resistant to rapamycin treatment (Fig. 1A, upper and middle panels, compare lanes 3 and 6). Under these conditions, the wild-type and mutant $p70^{s6k}$ proteins are expressed at comparable levels (Fig. 1A, lower panel). To ascertain whether $p70^{s6k}D_3E\text{-}E_{389}$ was capable of signalling downstream and whether it could confer rapamycin resistance on a putative target substrate, its effect on in vivo S6 phosphorylation was examined. However, there are two possible problems that could arise in considering an S6 reporter construct. First, such a construct would have to compete with endogenous S6 for assembly into ribosomes, and second, it would require an epitope tag, which could interfere with ribosome assembly and/or function. Earlier studies have shown that treatment of cells with rapamycin totally abolishes multiple S6 phosphorylation (10, 27). Therefore, it was reasoned that if the $p70^{s6k}D_3E\text{-}E_{389}$ construct protected S6 from rapamycin-induced dephosphorylation in a transient-transfection assay, a signal above the nontransfected background signal should be easily detected. Consequently, quiescent human 293 cells, transiently expressing the vector alone or the $p70^{s6k}D_3E\text{-}E_{389}$ mutant, were stimulated with insulin in the presence of $^{32}P_i$ with or without rapamycin. The level of S6 phosphorylation in total ribosomal proteins was monitored by autoradiography and quantified with a PhosphorImager following separation of the protein by SDS-PAGE and blotting onto a polyvinylidene difluoride membrane. In quiescent cells expressing the empty vector, no ^{32}P incorporation into S6 was detected, whereas insulin treatment led to a marked increase

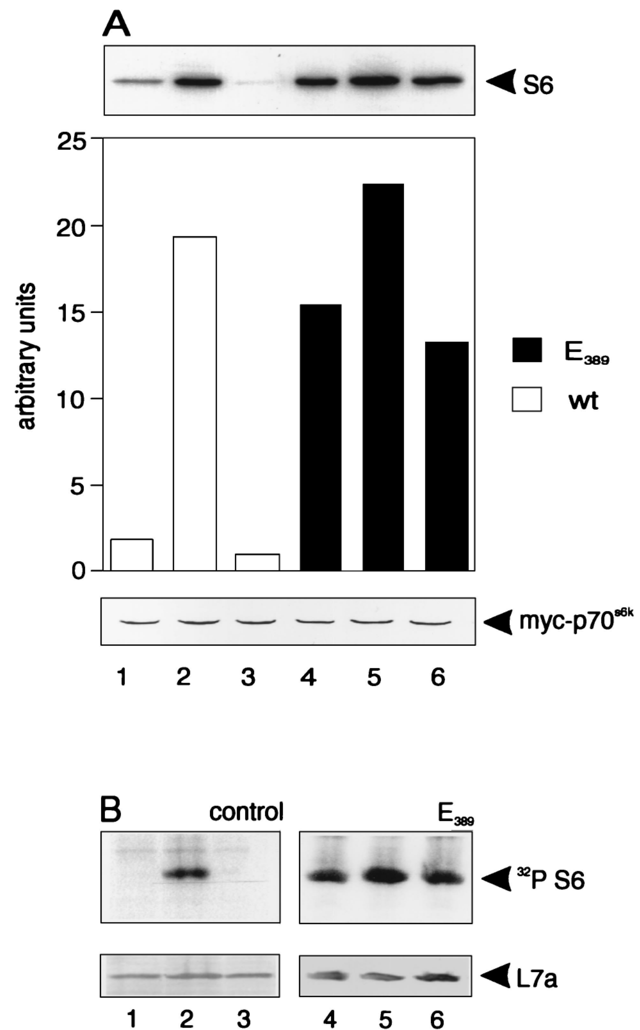

FIG. 1. The $p70^{s6k}D_3E\text{-}E_{389}$ mutant protects insulin-induced S6 phosphorylation from the effects of rapamycin. (A) Quiescent 293 cells transfected with either 10 μg of the myc-$p70^{s6k}$ construct (lanes 1 to 3) or the same amount of the rapamycin-resistant myc-$p70^{s6k}D_3E\text{-}E_{389}$ construct (lanes 4 to 6) were extracted directly (lanes 1 and 4) or first incubated for 15 min with the vehicle alone (lanes 2 and 5) or the vehicle plus 20 nM rapamycin (lanes 3 and 6) prior to stimulation with 1 μM insulin for 30 min. In vitro kinase assays were performed following immunoprecipitation with the anti-myc 9E10 antibody (upper panel). Kinase activity toward S6 (upper panel) was quantitated with a PhosphorImager after correction for expressed protein (lower panel) to yield specific activities, expressed in arbitrary units (middle panel). wt, wild type. (B) Quiescent 293 cells transfected with either 10 μg of the empty vector (lanes 1 to 3) or 10 μg of myc-$p70^{s6k}D_3E\text{-}E_{389}$ (lanes 4 to 6) were incubated for 1 h in the presence of $^{32}P_i$ and harvested directly (lanes 1 and 4) or after incubation for 30 min in the absence (lanes 2 and 5) or presence (lanes 3 and 6) of 20 nM rapamycin prior to stimulation with 1 μM insulin for 1 h. Total ribosomal proteins were resolved by SDS-PAGE, and ^{32}P-labelled S6 and the amount of 60S ribosomal protein L7a were analyzed as described in Materials and Methods. The data presented are representative of at least three independent experiments.

in the phosphorylation of the protein (Fig. 1B, upper panels, compare lanes 1 and 2). This effect was totally abolished by rapamycin pretreatment (Fig. 1B, upper panels, lane 3), consistent with the effect of the macrolide on either endogenous or ectopically expressed $p70^{s6k}$ activity (10, 19, 35, 55). In contrast to the endogenous kinase, the $p70^{s6k}D_3E\text{-}E_{389}$ mutant induced a high level of S6 phosphorylation in the absence of mitogens, which was further increased by insulin and largely resistant to rapamycin treatment (Fig. 1B, upper panel, lanes 4 to 6, re-

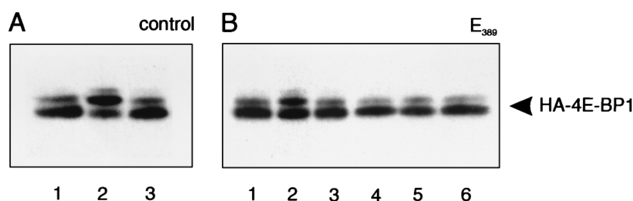

FIG. 2. The p70^{s6k}D$_3$E-E$_{389}$ mutant inhibits HA-4E-BP1 phosphorylation. Quiescent 293 cells were cotransfected with 2 μg of HA-4E-BP1 and 5 μg of the empty vector (A) or either 1 or 5 μg of the myc-p70^{s6k}D$_3$E-E$_{389}$ vector (B) (lanes 1 to 3 and 4 to 6, respectively). Quiescent cells were extracted directly (lanes 1 and 4) or first stimulated for 30 min with 1 μM insulin in the absence (lanes 2 and 5) or presence (lanes 3 and 6) of 20 nM rapamycin. Rapamycin was added 15 min prior to insulin. Detection of the HA-4E-BP1 was carried out by Western blot analysis with the anti-HA antibody 12CA5 after resolution of proteins by SDS-PAGE. The data presented are representative of three independent experiments.

spectively). The relative amount of ribosomal protein loaded on each lane (Fig. 1B, lower panels) was judged equivalent following quantification by fluorimetry with a polyclonal antibody against 60S ribosomal protein L7a (72). The insulin-induced increase in S6 phosphorylation, which is blocked by rapamycin, is most likely attributable to the endogenous kinase in nontransfected cells. The results demonstrate that the p70^{s6k}D$_3$E-E$_{389}$ rapamycin-resistant mutant is capable of signalling downstream in the presence of the macrolide and of conferring rapamycin resistance on S6, establishing the first direct link between S6 phosphorylation and p70^{s6k}.

The rapamycin-resistant p70^{s6k} mutant does not protect 4E-BP1 from dephosphorylation. To determine whether 4E-BP1 phosphorylation is mediated by p70^{s6k} or whether instead this event bifurcates at a point more proximal to the insulin receptor, an influenza virus HA epitope-tagged 4E-BP1 reporter construct, HA-4E-BP1, was transfected alone or with increasing amounts of the rapamycin-resistant p70^{s6k}D$_3$E-E$_{389}$ mutant. Insulin treatment of mitogen-deprived cells, transiently expressing only the HA-4E-BP1 construct, leads to increased phosphorylation of the protein, as judged by its decreased mobility, or band shift, on Western blots of cell extracts separated by SDS-PAGE (Fig. 2A, compare lanes 1 and 2). The insulin-induced band shift of HA-4E-BP1 is totally abolished in the presence of rapamycin (Fig. 2A, lane 3). In contrast to the ability of the rapamycin-resistant p70^{s6k}D$_3$E-E$_{389}$ mutant to induce S6 phosphorylation in the absence of insulin (Fig. 1B, lane 4), transient cotransfection of 293 cells with the same variant p70^{s6k} did not increase the basal phosphorylation level of cotransfected HA-4E-BP1 (compare Fig. 2B and A, lanes 1). On the contrary, the p70^{s6k}D$_3$E-E$_{389}$ mutant appeared to partially suppress the insulin-induced HA-4E-BP1 band shift (compare Fig. 2A and B, lanes 2) and, in addition, afforded no protection against rapamycin treatment (Fig. 2B, lane 3). The negative effect of the p70^{s6k}D$_3$E-E$_{389}$ mutant on insulin-induced 4E-BP1 phosphorylation was more pronounced when the amount of p70^{s6k} transfected was increased from 1 to 5 μg of cDNA (Fig. 2B, lanes 2 and 5). Not only did the p70^{s6k}D$_3$E-E$_{389}$ mutant block insulin-induced 4E-BP1 phosphorylation to the same extent as rapamycin (Fig. 2B, compare lanes 5 and 6), but it also lowered basal 4E-BP1 phosphorylation (Fig. 2B, compare lanes 1 and 4). Collectively, these results demonstrate that although the p70^{s6k}D$_3$E-E$_{389}$ mutant can protect S6 phosphorylation from the rapamycin-induced block, it does not protect 4E-BP1 phosphorylation from such treatment. Furthermore, the results show that overexpression of the rapamycin-resistant p70^{s6k} variant interferes with both basal and insulin-induced 4E-BP1 phosphorylation.

Overexpression of p70^{s6k} sequesters an upstream activator. The inhibitory effect of the p70^{s6k}D$_3$E-E$_{389}$ construct on insulin-induced 4E-BP1 phosphorylation was unexpected. It may be that overexpression of the mutant leads to the sequestering of an immediate upstream activator of 4E-BP1 and p70^{s6k} or that the effect of p70^{s6k}D$_3$E-E$_{389}$ on 4E-BP1 phosphorylation may be indirect, possibly owing to its high constitutive activity (Fig. 1A). To distinguish between these two alternatives, two differentially tagged wild-type p70^{s6k} constructs were utilized. Both constructs contained a myc epitope tag at their amino termini, and the second construct also had a GST tag at its carboxy terminus. The different molecular weights of the two constructs allowed their simultaneous detection on Western blots, whereas the GST tag enables the use of the second construct as a reporter for kinase activity. Thus, if an upstream activator is limiting, increasing the expression of the singly tagged wild-type kinase might be expected to block reporter myc-p70^{s6k}-GST activity by its ability to sequester the putative upstream activator. If, instead, the interfering effect is due to a unique feature of the p70^{s6k}D$_3$E-E$_{389}$ mutant, the wild-type p70^{s6k} should have no effect on reporter myc-p70^{s6k}-GST activation. However, as overexpression of the wild-type construct could raise total kinase activity, a myc-tagged kinase-dead p70^{s6k} was also examined. This variant, p70^{s6k}Q$_{100}$, was produced by mutating the essential lysine in the ATP binding pocket, as has been reported for other kinases (61). Cotransfection of either the wild-type or kinase-dead p70^{s6k} with the myc-p70^{s6k}-GST reporter blocked insulin-induced activation of the latter construct in a dose-dependent manner (Fig. 3A, upper and middle panels) without affecting reporter expression levels (Fig. 3A, lower panel). Identical results were obtained with the p70^{s6k}D$_3$E-E$_{389}$ mutant (data not shown). This interfering effect of both constructs was specific for p70^{s6k} because their overexpression had no effect on epidermal growth factor (EGF)-induced activation of a cotransfected HA-p44^{mapk} reporter construct in an immune complex assay employing myelin basic protein as a substrate (Fig. 3B). EGF was employed as the mitogen in these studies because insulin fails to activate the mitogen-activated protein kinase pathway in 293 cells (47, 68). To examine the effect of wild-type and kinase-dead p70^{s6k} proteins on downstream signalling, the ability of each construct to mediate endogenous S6 phosphorylation in transiently transfected 293 cells was monitored by ^{32}P incorporation into S6 as described above (Fig. 1B). Insulin-stimulated S6 phosphorylation was clearly repressed in quiescent cells transiently expressing kinase-dead p70^{s6k} (Fig. 3C, middle panels, lane 2), whereas the wild-type kinase continued to signal to S6 (Fig. 3C, middle panels, lane 5) at levels roughly equivalent to those of the endogenous kinase (Fig. 1B, lane 2). Indeed, in parallel transfection studies, we have found that expression of the wild-type construct, versus that of the empty vector, consistently raises basal S6 phosphorylation as well as insulin-stimulated S6 phosphorylation (data not shown). In contrast to that of the rapamycin-resistant mutant, overexpression of the wild-type construct did not protect S6 from the rapamycin-induced block in phosphorylation (compare Fig. 3C, middle panels, lane 6, and Fig. 1B, lane 6). Under these conditions, the kinase-dead and wild-type p70^{s6k} proteins were expressed equally (Fig. 3C, upper panels), and the loading of total ribosomal proteins was judged equivalent by fluorimetric analysis employing the polyclonal antibody against 60S ribosomal protein L7a (Fig. 3C, lower panels). Thus the kinase-dead construct performs as a legitimate dominant-interfering mutant, inhibiting both endogenous kinase activation and S6 phosphorylation. Although endogenous kinase activation, as measured by the myc-p70^{s6k}-GST reporter, is blocked by overexpression of the wild-type

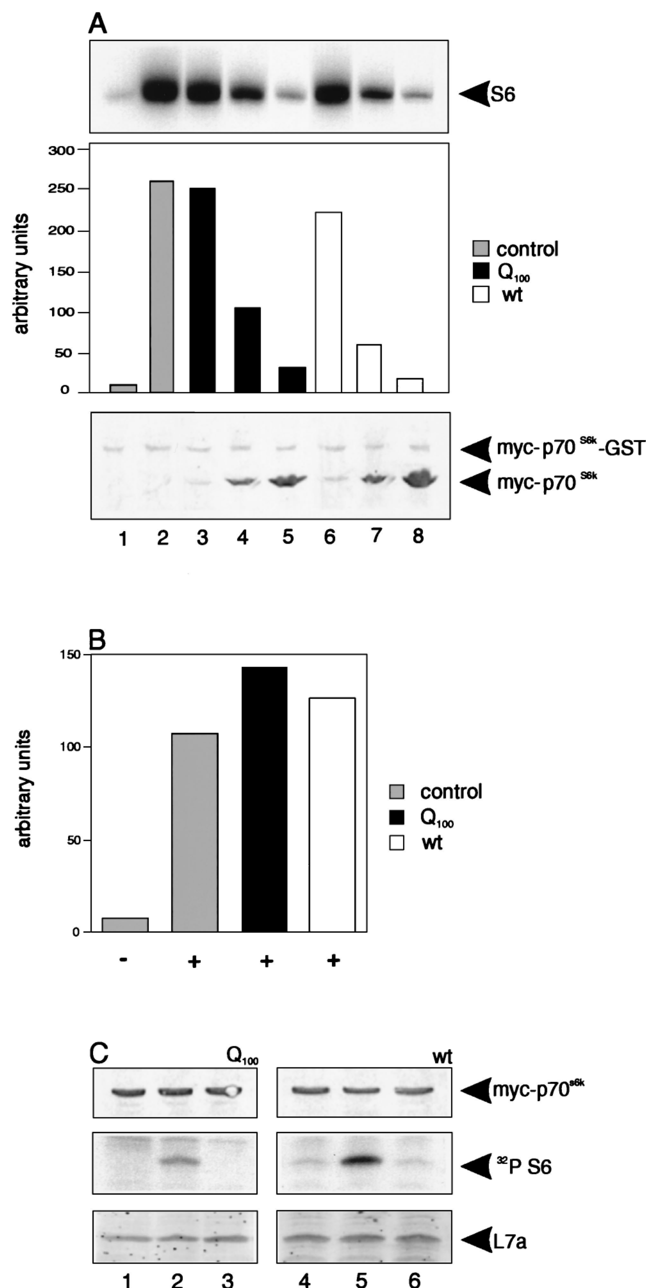

FIG. 3. Effect of wild-type and kinase-dead p70^{s6k} proteins on reporter kinase activity and S6 phosphorylation. (A) Quiescent 293 cells were cotransfected with 1 μg of reporter myc-p70^{s6k}-GST and either the empty vector (lanes 1 and 2) or 0.1 μg (lanes 3 and 6), 1 μg (lanes 4 and 7), or 5 μg (lanes 5 and 8) of the kinase-dead myc-p70^{s6k}Q$_{100}$ (lanes 3 to 5) or the wild-type (wt) myc-p70^{s6k} (lanes 6 to 8). Cells were extracted immediately (lane 1) or stimulated with 1 μM insulin for 30 min (lanes 2 to 8). The reporter myc-p70^{s6k}-GST activity was assayed for in vitro kinase activity (upper panel), and values were expressed as arbitrary units (middle panel) following correction for expression levels of reporter myc-p70^{s6k}-GST (lower panel) as described in Materials and Methods. The myc-p70^{s6k}Q$_{100}$ and wild-type myc-p70^{s6k} were visualized after Western blot analysis (see Materials and Methods). (B) Quiescent 293 cells, cotransfected with 2 μg of the reporter HA-p44^{mapk} construct and 5 μg of either the empty vector, the kinase-dead myc-p70^{s6k}Q$_{100}$, or the wild-type myc-p70^{s6k}, were treated with 5 nM EGF for 10 min as previously described (47). HA-p44^{mapk} activities were corrected as described above for reporter myc-p70^{s6k}-GST. − and +, no addition and addition of EGF, respectively. (C) Quiescent 293 cells transfected with either 10 μg of myc-p70^{s6k}Q$_{100}$ (lanes 1 to 3) or 10 μg of wild-type myc-p70^{s6k} (lanes 4 to 6) were incubated for 1 h in the presence of $^{32}P_i$ and harvested directly (lanes 1 and 4) or after incubation for 30 min in the presence of the vehicle (lanes 2 and 5) or the vehicle plus 20 nM rapamycin (lanes 3 and 6) prior to stimulation with 1 μM insulin for 1 h. Analysis of ^{32}P incorporated into S6 and the amount of L7a present in each fraction was as described for Fig. 1. myc-p70^{s6k}Q$_{100}$ and wild-type myc-p70^{s6k} were visualized as described above. The data presented are representative of three independent experiments, with the exception of the data in panel A, which are representative of two independent experiments.

kinase, this does not impair downstream signalling to S6, as total kinase activity is raised under these conditions (see Discussion). Taken together, the results suggest that overexpression of any of the three p70^{s6k} variants leads to the sequestering of a limiting upstream activator required for p70^{s6k} activation and possibly 4E-BP1 phosphorylation.

Overexpression of p70^{s6k} blocks 4E-BP1 phosphorylation and its release from eIF4E. The results described above indicate that the block observed in insulin-induced 4E-BP1 phosphorylation in the presence of the p70^{s6k}D$_3$E-E$_{389}$ mutant (Fig. 2B) was through the sequestering of a limiting upstream activator required for signalling to both proteins. To examine this possibility further, increasing amounts of the kinase-dead and wild-type p70^{s6k} proteins were coexpressed with a constant amount of the HA-4E-BP1 reporter. The results show that as the amount of the ectopically expressed kinase-dead p70^{s6k} is increased from 0.1 to 5 μg, the basal level of HA-4E-BP1 phosphorylation is reduced (Fig. 4A, lanes 1, 3, and 5). Likewise, the ability of insulin to induce HA-4E-BP1 phosphorylation is almost totally abrogated as the concentration of the kinase-dead p70^{s6k} is increased over the same range (Fig. 4A, lanes 2, 4, and 6). Qualitatively similar results were obtained by employing equivalent concentrations of the wild-type p70^{s6k} (Fig. 4B). In addition, analysis of endogenous 4E-BP1 phosphorylation by employing a specific 4E-BP1 antibody (22) revealed equivalent results (data not shown). Phosphorylation of 4E-BP1 disrupts its binding to eIF4E (53), allowing eIF4E to assemble in a productive mRNA cap-binding protein complex. If the 4E-BP1 phosphorylation sites blocked by both p70^{s6k} constructs are critical for function, they should prevent the release of 4E-BP1 from eIF4E. To test this possibility, extracts from cells expressing increasing amounts of the wild-type or kinase-dead p70^{s6k} were incubated with an aliquot of m^{7}GTP-coupled Sepharose, which specifically binds eIF4E. After extensive washing of the resin, bound proteins were eluted and resolved by SDS-PAGE. The amounts of bound eIF4E and HA-4E-BP1 present in each fraction were determined by Western blot analysis with specific antibodies for each protein. In extracts from quiescent cells transfected with the HA-4E-BP1 reporter alone, eIF4E and 4E-BP1 are clearly bound to the m^{7}GTP-coupled Sepharose (Fig. 4C, lane 1). In contrast, eluates from insulin-treated cell extracts contained an equivalent amount of eIF4E but no detectable HA-4E-BP1 (Fig. 4C, lane 2), consistent with earlier findings for endogenous 4E-BP1 (53) and increased phosphorylation of HA-4E-BP1 in response to insulin treatment (Fig. 2A). The presence of the cotransfected kinase-dead or wild-type p70^{s6k} did not alter 4E-BP1 binding to eIF4E in the absence of insulin (Fig. 4C, compare lanes 3 and 5 with lane 1). However, in the presence of either p70^{s6k} construct, insulin-induced release of eIF4E from the HA-4E-BP1 reporter is almost completely blocked (Fig. 4C, compare lanes 4 and 6 with lane 2). Thus, both the kinase-dead and wild-type p70^{s6k} proteins block 4E-BP1 phosphorylation and its release from eIF4E. The results suggest that each kinase construct is sequestering a common, limiting upstream activator of 4E-BP1 phosphorylation and that the signal transduction pathways leading to p70^{s6k} and 4E-BP1 phosphorylation appear to bifurcate immediately upstream of p70^{s6k}.

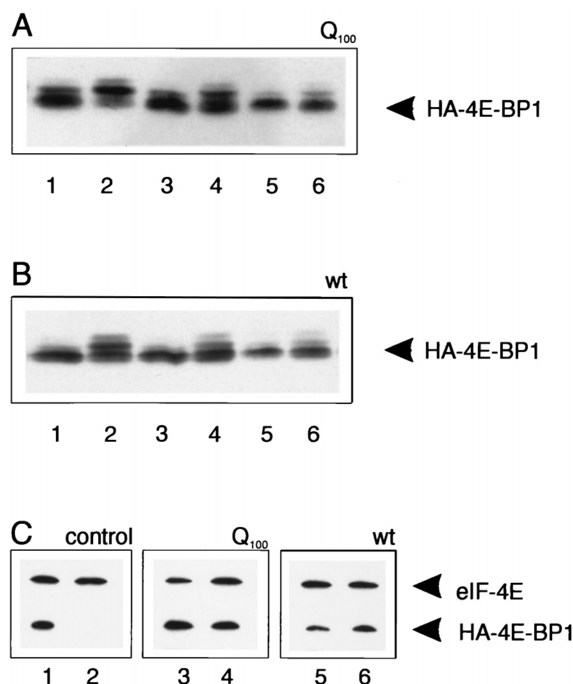

FIG. 4. Effect of wild-type and kinase-dead p70^{s6k} proteins on 4E-BP1 phosphorylation and its association with eIF4E. Quiescent 293 cells were cotransfected with 2 μg of HA-4E-BP1 and either 0.1 μg (lanes 1 and 2), 1 μg (lanes 3 and 4), or 5 μg (lanes 5 and 6) of the kinase-dead myc-p70$^{s6k}Q_{100}$ (A) or the wild-type (wt) myc-p70^{s6k} (B). Western blot analysis of HA-4E-BP1 was conducted as described for Fig. 2 following no treatment (lanes 1, 3, and 5) or stimulation with 1 μM insulin for 30 min (lanes 2, 4, and 6). (C) Extracts from quiescent (lanes 1, 3, and 5) or insulin-stimulated (lanes 2, 4, and 6) 293 cells, transfected with 5 μg of either the empty vector (lanes 1 and 2), myc-p70$^{s6k}Q_{100}$ (lanes 3 and 4), or wild-type myc-p70^{s6k} (lanes 5 and 6), were incubated with m^7GTP-Sepharose as described in Materials and Methods. eIF4E or HA-4E-BP1 bound to the initiation factor was detected by Western blot analysis with the appropriate antibodies (see Materials and Methods). The data presented are representative of three independent experiments.

Rapamycin and dominant-interfering p70^{s6k} block the same set of phosphorylation sites in p70^{s6k} and 4E-BP1. Rapamycin blocks p70^{s6k} activation as well as increased S6 and 4E-BP1 phosphorylation. In the case of p70^{s6k}, this effect is exerted principally through the dephosphorylation of T_{389}, consistent with substitution of an acidic residue at this position conferring rapamycin resistance on p70^{s6k} activity and S6 phosphorylation (Fig. 1A and B, respectively). Since the kinase-dead p70^{s6k} also interferes with reporter p70^{s6k} activation (Fig. 3A), as well as S6 (Fig. 3C) and 4E-BP1 (Fig. 4A) phosphorylation, this raises the possibility that the kinase-dead p70^{s6k} blocks the same signalling event that is obstructed by rapamycin. To test this possibility, two-dimensional tryptic-chymotryptic phosphopeptide maps of either myc-p70^{s6k}-GST or HA-4E-BP1 were analyzed. In agreement with earlier observations with endogenous and overexpressed p70^{s6k} (13, 24, 54), addition of rapamycin selectively blocked insulin-induced phosphorylation of T_{229}, T_{389}, and S_{404} and suppressed phosphorylation of S_{411} (compare Fig. 5A and B). Coexpression with the kinase-dead construct, myc-p70$^{s6k}Q_{100}$, preferentially blocked the same set of phosphorylation sites as rapamycin with the myc-p70^{s6k}-GST reporter construct (compare Fig. 5C and B), supporting the possibility that these inhibitory effects may be exerted through a common target. Identical results were obtained when the wild-type kinase or the p70$^{s6k}D_3E$-E_{389} mutant was overexpressed with the myc-p70^{s6k}-GST reporter (data not shown). In the case of 4E-BP1, one principal site of phosphorylation has been identified, S_{64}, whose phosphorylation has been implicated in disrupting 4E-BP1 binding with eIF4E (40). In agreement with the band shift displayed by HA-4E-BP1 in response to insulin treatment (Fig. 2A), in parallel insulin induces a three- to fourfold increase in the amount of phosphate incorporated into the protein (Fig. 6, compare bars 1 and 2). Pretreatment with rapamycin or coexpression of kinase-dead p70^{s6k} significantly lowers the amount of phosphate incorporated into HA-4E-BP1 (Fig. 6, bars 3 and 4, respectively). Analysis of ^{32}P-labelled HA-4E-BP1 from insulin-treated cells revealed a large number of phosphopeptides, which have been

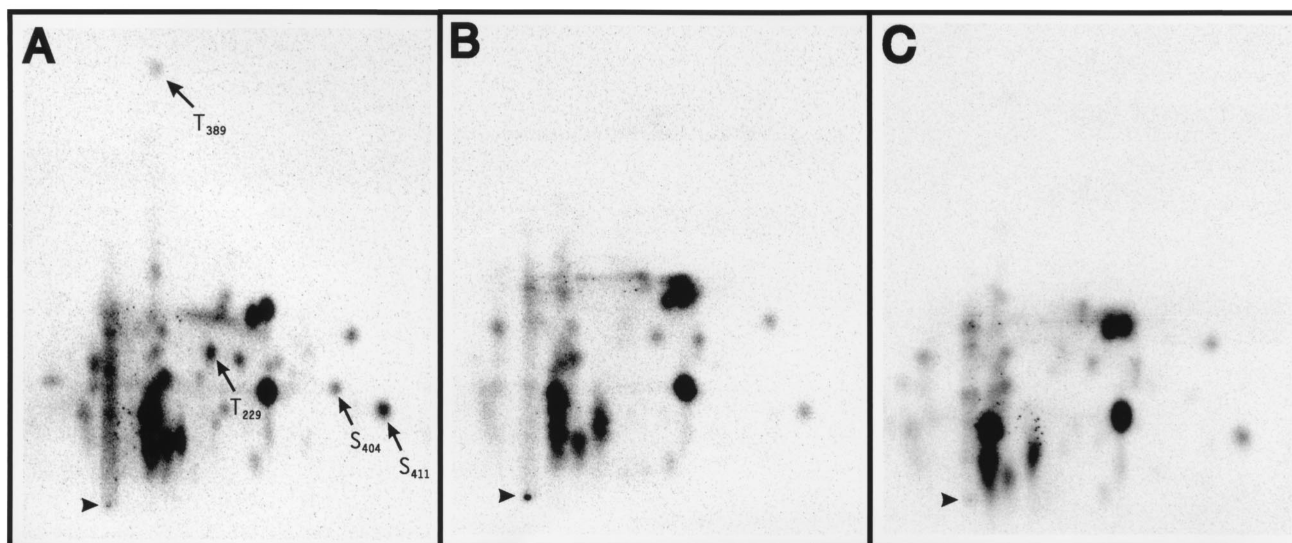

FIG. 5. Effect of kinase-dead p70^{s6k} and rapamycin on reporter p70^{s6k} phosphorylation sites. Quiescent 293 cells transfected with 1 μg of reporter myc-p70^{s6k}-GST were stimulated with 1 μM insulin for 30 min in the absence (A) or presence (B) of 20 nM rapamycin added 15 min prior to the addition of insulin. (C) Quiescent 293 cells cotransfected with 1 μg of reporter myc-p70^{s6k}-GST and 5 μg of myc-p70$^{s6k}Q_{100}$ were stimulated with 1 μM insulin for 30 min. Two-dimensional phosphopeptide mapping of myc-p70^{s6k}-GST was carried out as described in Materials and Methods. Arrowheads and arrows indicate the origin of sample application and the locations of the peptides containing the affected phosphorylation sites. The data are representative of two independent experiments.

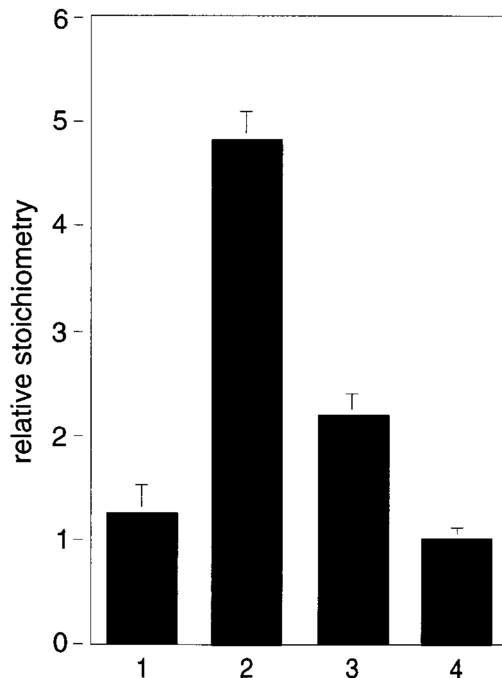

FIG. 6. Relative stoichiometries of HA-4E-BP1 phosphorylation. 293 cells were transiently transfected with 2 μg of HA-4E-BP1, serum starved, and labelled with $^{32}P_i$ as described in Materials and Methods. The cells were then extracted directly (bar 1), after treatment with 1 μM insulin for 30 min (bar 2), after pretreatment with 20 nM rapamycin for 15 min followed by treatment with 1 μM insulin for 30 min (bar 3), or after treatment with 5 μg of cotransfected myc-p70^{s6k} Q_{100} and 1 μM insulin treatment for 30 min (bar 4). The HA-4E-BP1 constructs were immunoprecipitated and processed as described in Materials and Methods. Relative stoichiometries are expressed in arbitrary units as the ratio of phosphate incorporated into HA-4E-BP1. Error bars indicate standard deviations.

tentatively designated a through h (Fig. 7A). Pretreatment with rapamycin strongly suppresses the phosphorylation of peptides a, b, e, and f but has little effect on peptides c, d, g, and h (Fig. 7B). As with reporter p70^{s6k} (Fig. 5C), the kinase-dead construct suppresses the same set of phosphorylation sites in HA-4E-BP1 as rapamycin, leaving the remaining sites unaffected (Fig. 7C). Similar results were obtained with insulin and rapamycin when endogenous 4E-BP1 was analyzed (unpublished data). As many of the phosphopeptides derived from 4E-BP1 include phosphothreonine, either alone or together with phosphoserine (data not shown), and insulin induction leads to the appearance of multiple band shifts (Fig. 2 and 4), the results imply that regulation of 4E-BP1 may be more complicated and not limited to just the phosphorylation of S_{64}. The patterns of inhibition exerted by the kinase-dead construct and rapamycin are equivalent, supporting the hypothesis that the point of bifurcation between p70^{s6k} and 4E-BP1 is immediately upstream of p70^{s6k} and that the proposed limiting upstream activator sequestered by the p70^{s6k} constructs is rapamycin sensitive.

DISCUSSION

Here we have employed three different p70^{s6k} variants to establish the role of the kinase in signalling to S6 and to demonstrate that neither 4E-BP1 nor a 4E-BP1 kinase is a downstream target of p70^{s6k}. As depicted in the model in Fig. 8, all three p70^{s6k} constructs share the ability to block signalling to 4E-BP1, apparently by sequestering a common upstream activator, suggesting that the 4E-BP1 pathway bifurcates at a point immediately upstream of p70^{s6k}.

The role of p70^{s6k} as the upstream mediator of in vivo S6 phosphorylation was initially deduced from its ability to phosphorylate the same sites on S6 in vitro (17) as were observed in vivo (3, 34) with better kinetics than that measured for the other mitogen-activated S6 kinase, p90^{rsk} (36, 67). This hypothesis was further supported by the observation that rapamycin blocked p70^{s6k} activation and S6 phosphorylation without affecting p90^{rsk} activation (10, 35, 55). The data presented here establish p70^{s6k} as the in vivo S6 kinase. First, a rapamycin-resistant form of p70^{s6k} rescues the inhibitory effects of rapamycin on S6 phosphorylation (Fig. 1B), and its high basal kinase activity is sufficient to increase S6 phosphorylation in the absence of insulin (Fig. 1B). In agreement with this obser-

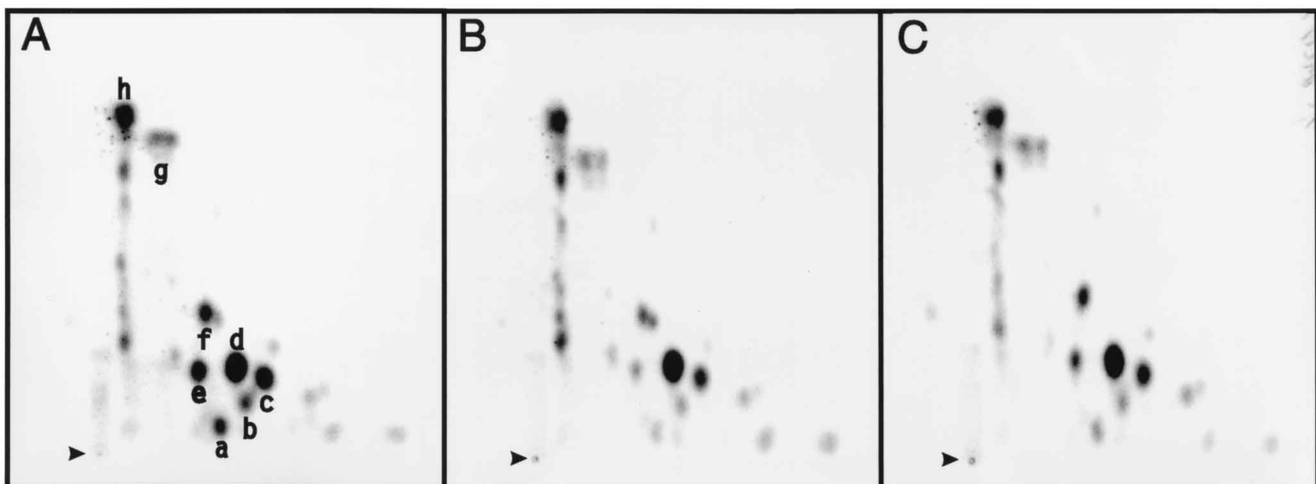

FIG. 7. Effect of kinase-dead p70^{s6k} and rapamycin on reporter 4E-BP1 phosphorylation sites. Quiescent 293 cells transfected with 2 μg of reporter HA-4E-BP1 were stimulated with 1 μM insulin for 30 min in the absence (A) or presence (B) of 20 nM rapamycin added 15 min prior to the addition of insulin. (C) Quiescent 293 cells cotransfected with 2 μg of reporter HA-4E-BP1 and 5 μg of myc-p70$^{s6k}Q_{100}$ were stimulated with 1 μM insulin for 30 min. Two-dimensional phosphopeptide mapping of HA-4E-BP1 was carried out as described in Materials and Methods. Arrowheads indicate the origin of sample application, and the major phosphopeptides are labelled a to h. The data are representative of two independent experiments.

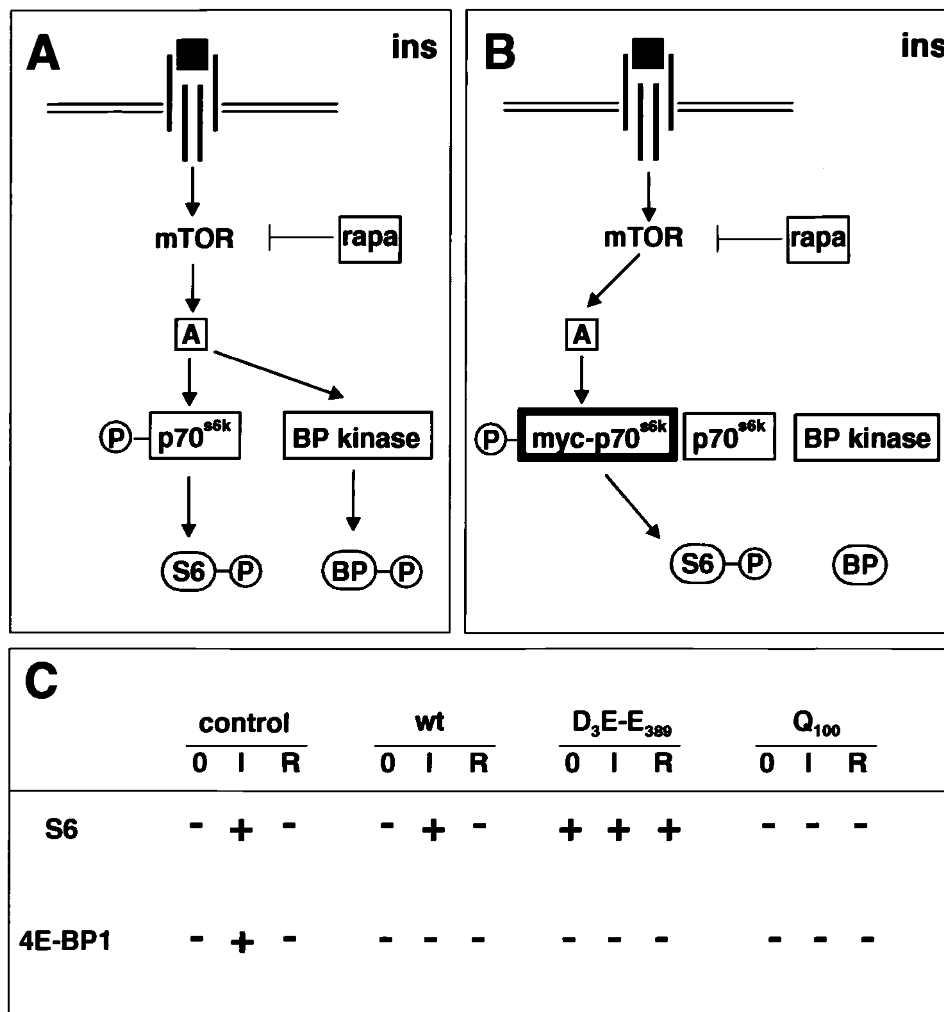

FIG. 8. Schematic representations of the FRAP-p70^{s6k} pathways for untransfected insulin (ins)-stimulated cells (A) and for cells stimulated with insulin and transfected with myc-tagged p70^{s6k} (myc-p70^{s6k}) (B) are shown. The putative upstream activator (box A), shared by p70^{s6k} and the hypothetical 4E-BP1 kinase (BP kinase), as well as the phosphorylation states of endogenous p70^{s6k}, S6, and 4E-BP1 (BP) are indicated. The overexpressed myc-p70^{s6k} is depicted in boldface outline. The inhibitory effect of rapamycin (rapa) on mTOR/FRAP (mTOR) is shown. (C) Summary of the effects of cotransfected p70^{s6k} constructs (control, cells transfected with the empty vector; wt, wild-type p70^{s6k}; D_3E-E_{389}, rapamycin-resistant mutant; Q_{100}, kinase-dead p70^{s6k}) on the phosphorylation/activation state of S6 and HA-4E-BP1. −, hypophosphorylation in the case of 4E-BP1 and no phosphorylation for S6; +, hyperphosphorylation of 4E-BP1 and S6.

vation, the kinase-dead p70^{s6k} construct suppresses S6 phosphorylation in response to insulin treatment (Fig. 3C), consistent with its ability to inhibit the activation of the reporter myc-p70^{s6k}-GST (Fig. 3A) by blocking phosphorylation of T_{389} (Fig. 5C). Finally, ectopic expression of the wild-type kinase leads to slightly elevated S6 kinase activity in the absence of insulin, as reflected in the higher basal level of S6 phosphorylation (Fig. 3C). This construct also maintains its sensitivity to rapamycin and its ability to signal to S6 (Fig. 3C), despite inhibiting activation of the reporter myc-p70^{s6k}-GST (Fig. 3A). This is most likely due to the fact that even though the specific activities of both the reporter myc-p70^{s6k}-GST and the cotransfected wild-type myc-p70^{s6k} are decreasing as a function of increasing amounts of cotransfected myc-p70^{s6k} (Fig. 3A), total p70^{s6k} activity is actually higher at the concentrations of the construct which were transfected. Indeed, as a function of increased expression of the wild-type p70^{s6k}, there is an initial augmentation in total p70^{s6k} activity, which is diminished at higher levels of expression but still significantly greater than that in nontransfected cells (data not shown). Thus, it is possible to titrate the upstream activator without preventing downstream signalling through p70^{s6k} (Fig. 8B). Taken together, the data establish S6 as a downstream target of p70^{s6k} and underscore the importance of the p70^{s6k} constructs as tools for determining the validity of other potential substrates.

The ability of all three p70^{s6k} constructs to block signalling to p70^{s6k} (Fig. 8B) as well as 4E-BP1 (Fig. 8C) indicates that the limiting upstream activator is common to both pathways and functions as a bifurcation point (Fig. 8A). From the phosphopeptide maps of p70^{s6k} and 4E-BP1, derived from cells either treated with rapamycin or coexpressing the kinase-dead p70^{s6k} construct (Fig. 5 and 7), it appears that the common upstream activator is also rapamycin sensitive (Fig. 8). An obvious candidate would be mTOR/FRAP, the inhibitory target of the rapamycin-FKBP12 complex (6, 60). mTOR/FRAP has the capacity to autophosphorylate (6, 9). This observation and the finding that a rapamycin-resistant mTOR/FRAP mutant protects a reporter p70^{s6k} construct from inactivation by the macrolide has led to the suggestion that mTOR/FRAP may be an upstream p70^{s6k} kinase (7). However, attempts to copre-

cipitate p70^{s6k} and mTOR/FRAP, or to show that mTOR/FRAP phosphorylates p70^{s6k}, have been unsuccessful (reference 7 and data not shown). Indeed, a p70^{s6k} variant which lacks the first 54 and last 104 amino acids, termed p70$^{s6k}\Delta N_{54}\Delta C_{104}$, has recently been described (13). This variant displays low basal activity, is activated by mitogenic treatment, and is completely resistant to rapamycin. Consistent with its resistance to rapamycin, there is no effect of the macrolide on the phosphorylation of the rapamycin-sensitive p70^{s6k} sites, T_{229} and T_{389}. These findings, combined with additional observations, led to the hypothesis that rapamycin does not inhibit a T_{229} or T_{389} kinase but appears to act on an effector molecule which regulates p70^{s6k} activity through the amino terminus (13). At this point, it is not possible to determine whether this effector molecule has a negative or positive effect on p70^{s6k} activation or whether its activity is regulated by mitogens.

Initially, it was reported that 4E-BP1 was a direct downstream target of the insulin-induced mitogen-activated protein kinase (40). The subsequent observation that 4E-BP1 phosphorylation, like p70^{s6k} activation, is rapamycin sensitive indicated that instead 4E-BP1 phosphorylation resides on the same signal transduction pathway as p70^{s6k} (4, 68). Here, two-dimensional phosphopeptide mapping of 4E-BP1 revealed two sets of phosphorylation sites. One set is highly phosphorylated in the absence of mitogens and does not result in a band shift (unpublished data), as reflected by the high stoichiometry of 4E-BP1 phosphorylation in the basal state (Fig. 6). The second set is characterized by an acute increase in phosphorylation in response to insulin, which is partially blocked in the presence of rapamycin. Thus, none of the sites observed, including the second set of sites, are completely blocked by treatment with rapamycin. However, rapamycin treatment abolishes mTOR/FRAP autophosphorylation (7, 9) and, therefore, would presumably abolish the ability of mTOR/FRAP to phosphorylate any downstream substrate. This observation suggests either that mTOR/FRAP is not a 4E-BP1 kinase or that other kinases are also capable of phosphorylating the same set of sites in 4E-BP1. In addition, despite the fact that coexpression with p70^{s6k} blocks the same set of 4E-BP1 phosphorylation sites as rapamycin (Fig. 7), the 4E-BP1 kinase which modifies the mitogen-upregulated sites does not appear to be equivalent to the T_{389} and T_{229} p70^{s6k} kinase(s). This is because the motifs flanking possible 4E-BP1 phosphorylation sites (53) are distinct from the rapamycin-sensitive p70^{s6k} sites, with the possible exception of S_{411} (54). Furthermore, the sites affected in the two proteins display strikingly different sensitivities to rapamycin (Fig. 5 and 7). Nevertheless, the 4E-BP1 kinase appears to be subject to regulation by the same putative rapamycin-sensitive effector molecule mentioned above. Such a model would also be consistent with the preliminary observation that overexpression of 4E-BP1 does not affect p70^{s6k} activation (unpublished data), as overexpression of 4E-BP1 might be capable of sequestering the 4E-BP1 kinase but not the common upstream activator (Fig. 8A). It will be important to identify the in vivo 4E-BP1 phosphorylation sites in order to obtain some insight with regard to possible kinases involved in basal and insulin-stimulated phosphorylation.

In addition to inhibiting p70^{s6k} activation, as well as S6 and 4E-BP1 phosphorylation, rapamycin also suppresses the selective translational upregulation of an essential family of mRNAs bearing an oligopyrimidine tract at their transcriptional start, collectively termed 5′TOP mRNAs. These transcripts can represent up to 20% of the total mRNA in the cell and code for many components of the translational apparatus, most notably ribosomal proteins (46). In cultured quiescent cells, these transcripts are distributed between monosomes, disomes, and messenger RNP particles, from which they selectively redistribute to large polysomes upon mitogen stimulation (28). Disruption of the 5′TOP, by substitution of purines for pyrimidines, is sufficient to induce these transcripts to redistribute to polysomes in the absence of the mitogen (1). Thus, this motif appears to act as a translational suppressor. It has been suggested that upregulation of these transcripts may be under the control of 4E-BP1 (41); however, in cells overexpressing eIF4E, upregulation of 5′TOP mRNAs still requires mitogen stimulation (62). Indeed, eIF4E, though implicated in the general upregulation of translation (65), appears to be especially important in the translation of a small subset of mRNAs which contain highly structured 5′ untranslated regions, such as ornithine decarboxylase (43, 63), ornithine aminotransferase (16), cyclin D1 (59), Q23 or p23 (5), and myc (12). Consistent with this observation and the data presented here, we have recently demonstrated that a dominant-interfering p70^{s6k} and a rapamycin-resistant p70^{s6k} either suppressed 5′TOP translation or prevented rapamycin-induced runoff of these transcripts from polysomes, respectively (28a). Given the specificity of p70^{s6k} for S6 and the location of the ribosomal protein at or near the 40S ribosomal mRNA binding site (51), the prediction would be that the effects of p70^{s6k} are mediated through S6 phosphorylation, a model presently under examination.

The impact of rapamycin on the translational machinery has led to the suggestion that this pathway may be dedicated to the control of protein synthesis (68). Besides S6 and 4E-BP1, insulin-induced phosphorylation of eIF4E (45) and dephosphorylation of eEF2 (57) have been recently implicated as potential downstream targets of this pathway. The phosphorylation of eIF4E is thought to be mediated by protein kinase C (29, 70). When bound to 4E-BP1, eIF4E does not serve as a substrate for protein kinase C (70). In contrast to that of eIF4E, eEF2 phosphorylation is known to be regulated by the Ca^{2+}-calmodulin-dependent protein kinase III (49); however, the mechanism by which rapamycin blocks this response is unknown (57). Despite the apparent importance of 4E-BP1 and eIF4E phosphorylation, as well as eEF2 dephosphorylation, to general protein synthesis, it should be noted that in many cell types rapamycin has only a partial effect on the rate of global translation (11, 27, 30, 45). In establishing the relative importance of these phosphorylation events, it will be important to understand the signalling pathways operating in each cell type as well as the possible involvement of other translational components. Although inhibitors, such as rapamycin, have been valuable in pointing to potential targets, future studies will demand more precise tools. The p70^{s6k} variants described here have been useful in resolving the point of bifurcation of 4E-BP1 and p70^{s6k} phosphorylation as well as the functional importance of p70^{s6k} activation in 5′TOP mRNA expression (28a). These tools undoubtedly will be effective in resolving the role of p70^{s6k} in signalling to other rapamycin-sensitive targets, such as eEF2. Given that the p70^{s6k} constructs are capable of titrating an upstream activator which controls p70^{s6k} activation and 4E-BP1 phosphorylation, they should also prove to be powerful reagents for identifying such an upstream signalling component. The identification of this molecule will increase our understanding of the mechanisms by which insulin and other extracellular stimuli regulate translation.

ACKNOWLEDGMENTS

We thank C. Hamilton for typing the manuscript, M. Rothnie for help with the figures, and F. Zilbermann for assistance with tissue culture. We also thank B. Hemmings, H. Lane, and J. Montagne for critically reading the manuscript. S. C. Kozma kindly provided the

myc-p70^{s6k}-Q_{100} construct, the HA-p44^{mapk} construct was a gift from J. Pouysségur, and the L7a antibody was generously provided by A. Ziemiecki.

S.R.V.M. and A.-C.G. are recipients of predoctoral fellowships from Boehringer Ingelheim Fonds and the National Science and Engineering Research Council of Canada, respectively. P.B.D. and N.P. are recipients of long-term postdoctoral fellowships from the Human Frontier Science Program Organization and the European Molecular Biology Organization, respectively. These studies were supported in part by a grant from the Human Frontier Science Program Organization (to G.T. and N.S.).

S. R. von Manteuffel and P. B. Dennis contributed equally to this work.

REFERENCES

1. **Avni, D., S. Shama, F. Loreni, and O. Meyuhas.** 1994. Vertebrate mRNAs with a 5′-terminal pyrimidine tract are candidates for translational repression in quiescent cells: characterization of the translational *cis*-regulatory element. Mol. Cell. Biol. **14:**3822–3833.
2. **Azpiazu, I., A. R. Saltiel, A. A. DePaoli-Roach, and J. C. Lawrence, Jr.** 1996. Regulation of both glycogen synthase and PHAS-I by insulin in rat skeletal muscle involves mitogen-activated protein kinase-independent and rapamycin-sensitive pathways. J. Biol. Chem. **271:**5033–5039.
3. **Bandi, H. R., S. Ferrari, J. Krieg, H. E. Meyer, and G. Thomas.** 1993. Identification of 40S ribosomal protein S6 phosphorylation sites in Swiss mouse 3T3 fibroblasts stimulated with serum. J. Biol. Chem. **268:**4530–4533.
4. **Beretta, L., A.-C. Gingras, Y. V. Svitkin, M. N. Hall, and N. Sonenberg.** 1996. Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. EMBO J. **15:**658–664.
5. **Bommer, U. A., A. Lazaris-Karatzas, A. DeBenedetti, P. Nurnberg, R. Benndorf, H. Bielka, and N. Sonenberg.** 1994. Translational regulation of the mammalian growth-related protein P23: involvement of eIF-4E. Cell. Mol. Biol. Res. **40:**633–641.
6. **Brown, E. J., M. A. Albers, T. B. Shin, K. Ichikawa, C. T. Keith, W. S. Lane, and S. L. Schreiber.** 1994. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature **369:**756–758.
7. **Brown, E. J., P. A. Beal, C. T. Keith, J. Chen, T. B. Shin, and S. L. Schreiber.** 1995. Control of p70 S6 kinase by kinase activity of FRAP *in vivo*. Nature **377:**441–446.
8. **Brown, E. J., and S. L. Schreiber.** 1996. A signalling pathway to translational control. Cell **86:**517–520.
9. **Brunn, G. J., J. Williams, C. Sabers, G. Wiederrecht, J. C. Lawrence, Jr., and R. T. Abraham.** 1996. Direct inhibition of the signalling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002. EMBO J. **15:**5256–5267.
10. **Chung, J., C. J. Kuo, G. R. Crabtree, and J. Blenis.** 1992. Rapamycin-FKBP specifically blocks growth-dependent activation of and signalling by the 70 kd S6 protein kinases. Cell **69:**1227–1236.
11. **Dardevet, D., C. Sornet, T. Vary, and J. Grizard.** 1996. Phosphatidylinositol 3-kinase and p70 S6 kinase participate in the regulation of protein turnover in skeletal muscle by insulin and insulin-like growth factor I. Endocrinology **137:**4087–4094.
12. **DeBenedetti, A., B. Joshi, J. R. Graff, and S. G. Zimmer.** 1994. CHO cells transformed by the translation factor eIF4E display increased c-myc expression but require overexpression of Max for tumorigenicity. Mol. Cell. Differ. **2:**347–371.
13. **Dennis, P. B., N. Pullen, S. C. Kozma, and G. Thomas.** 1996. The principal rapamycin-sensitive p70^{s6k} phosphorylation sites T_{229} and T_{389} are differentially regulated by rapamycin-insensitive kinase-kinases. Mol. Cell. Biol. **16:** 6242–6251.
14. **DePhilip, R. M., W. A. Rudert, and I. Lieberman.** 1980. Preferential stimulation of ribosomal protein synthesis by insulin and in the absence of ribosomal and messenger ribonucleic acid formation. Biochemistry **19:**1662–1669.
15. **de Vries-Smits, A. M. M., B. M. T. Burgering, S. J. Leevers, C. J. Marshall, and J. L. Bos.** 1992. Involvement of p21^{ras} in activation of extracellular signal-regulated kinase 2. Nature **357:**602–604.
16. **Fagan, R. J., A. Lazaris-Karatzas, N. Sonenberg, and R. Rozen.** 1991. Translational control of ornithine aminotransferase: modulation by initiation factor eIF-4E. J. Biol. Chem. **266:**16518–16523.
17. **Ferrari, S., H. R. Bandi, B. M. Bussian, and G. Thomas.** 1991. Mitogen-activated 70K S6 kinase. J. Biol. Chem. **266:**22770–22775.
18. **Ferrari, S., W. Bannwarth, S. J. Morley, N. F. Totty, and G. Thomas.** 1992. Activation of p70^{s6k} is associated with phosphorylation of four clustered sites displaying Ser/Thr-Pro motifs. Proc. Natl. Acad. Sci. USA **89:**7282–7285.
19. **Ferrari, S., R. B. Pearson, M. Siegmann, S. C. Kozma, and G. Thomas.** 1993. The immunosuppressant rapamycin induces inactivation of p70^{s6k} through dephosphorylation of a novel set of sites. J. Biol. Chem. **268:**16091–16094.
20. **Ferrari, S., and G. Thomas.** 1994. S6 phosphorylation and the p70^{s6k}/p85^{s6k}. Crit. Rev. Biochem. Mol. Biol. **29:**385–413.
21. **Flotow, H., and G. Thomas.** 1992. Substrate recognition determinants of the mitogen-activated 70K S6 kinase from rat liver. J. Biol. Chem. **267:**3074–3078.
22. **Gingras, A.-C., Y. Svitkin, G. J. Belsham, A. Pause, and N. Sonenberg.** 1996. Activation of the translational suppressor 4E-BP1 following infection with encephalomyocarditis virus and poliovirus. Proc. Natl. Acad. Sci. USA **93:** 5578–5583.
23. **Hammond, M., and L. H. Bowman.** 1988. Insulin stimulates the translation of ribosomal proteins and the transcription of rDNA in mouse myoblasts. J. Biol. Chem. **263:**17785–17791.
24. **Han, J. W., R. B. Pearson, P. B. Dennis, and G. Thomas.** 1995. Rapamycin, wortmannin, and the methylxanthine SQ20006 inactivate p70^{s6k} by inducing dephosphorylation of the same subset of sites. J. Biol. Chem. **270:**21396–21403.
25. **Haystead, T. A. J., C. M. M. Haystead, C. Hu, T. A. Lin, and J. C. Lawrence, Jr.** 1994. Phosphorylation of PHAS-I by mitogen-activated protein (MAP) kinase. J. Biol. Chem. **269:**23185–23191.
26. **Hunter, T.** 1995. When is a lipid kinase not a lipid kinase? When it is a protein kinase. Cell **83:**1–4.
27. **Jefferies, H. B. J., C. Reinhard, S. C. Kozma, and G. Thomas.** 1994. Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family. Proc. Natl. Acad. Sci. USA **91:**4441–4445.
28. **Jefferies, H. B. J., G. Thomas, and G. Thomas.** 1994. Elongation factor-1a mRNA is selectively translated following mitogenic stimulation. J. Biol. Chem. **269:**4367–4372.

28a. **Jefferies, H. B. J., S. Fumagalli, P. B. Dennis, D. Reinhard, R. B. Pearson, and G. Thomas.** 1997. Rapamycin suppresses 5′TOp mRNA translation through inhibition of p70^{s6k}, EMBO J. **16:**3693–3704.

29. **Joshi, B., A.-L. Cai, B. D. Keiper, W. B. Minich, R. Mendez, C. M. Beach, J. Stepinski, R. Stolarski, E. Darzynkiewicz, and R. E. Rhoads.** 1995. Phosphorylation of eukaryotic protein synthesis initiation factor 4E at Ser-209. J. Biol. Chem. **270:**14597–14603.
30. **Kardalinou, E., N. Zhelev, C. A. Hazzalin, and L. C. Mahadevan.** 1994. Anisomycin and rapamycin define an area upstream of p70/85^{S6k} containing a bifurcation to histone H3–HMG-like protein phosphorylation and c-*fos*–c-*jun* induction. Mol. Cell. Biol. **14:**1066–1074.
31. **Kato, J. Y., M. Matsuoka, K. Polyak, J. Massague, and C. J. Sherr.** 1994. Cyclic AMP-induced G_1 phase arrest mediated by an inhibitor (p27^{kip1}) of cyclin-dependent kinase 4 activation. Cell **79:**487–496.
32. **Kolodziej, P. A., and R. A. Young.** 1991. Epitope tagging and protein surveillance. Methods Enzymol. **194:**508–519.
33. **Krieg, J., J. Hofsteenge, and G. Thomas.** 1988. Identification of the 40S ribosomal protein S6 phosphorylation sites induced by cycloheximide. J. Biol. Chem. **263:**11473–11477.
34. **Krieg, J., A. R. Olivier, and G. Thomas.** 1988. Analysis of 40S ribosomal protein S6 phosphorylation during the mitogenic response. Methods Enzymol. **164:**575.
35. **Kuo, C. J., J. Chung, D. F. Fiorentino, W. M. Flanagan, J. Blenis, and G. R. Crabtree.** 1992. Rapamycin selectively inhibits interleukin-2 activation of p70 S6 kinase. Nature **358:**70–73.
36. **Kyriakis, J. M., and J. Avruch.** 1994. S6 kinases and MAP kinases: sequential intermediates in insulin/mitogen-activated protein kinase cascades, p. 84–184. *In* J. R. Woodgett (ed.), Protein kinases. Oxford University Press, Oxford, United Kingdom.
37. **Lane, H. A., A. Fernandez, N. J. C. Lamb, and G. Thomas.** 1993. p70^{s6k} function is essential for G1 progression. Nature **363:**170–172.
38. **Lane, H. A., S. J. Morley, M. Doree, S. C. Kozma, and G. Thomas.** 1992. Identification and early activation of a Xenopus laevis p70^{s6k} following progesterone-induced meiotic maturation. EMBO J. **11:**1743–1749.
39. **Lane, H. A., and G. Thomas.** 1991. Purification and properties of mitogen-activated S6 kinase from rat liver and 3T3 cells. Methods Enzymol. **200:**269–291.
40. **Lin, T., X. Kong, T. A. J. Haystead, A. Pause, G. Belsham, N. Sonenberg, and J. C. Lawrence, Jr.** 1994. PHAS-I as a link between mitogen-activated protein kinase and translation initiation. Science **266:**653–656.
41. **Lin, T. A., X. Kong, A. R. Saltiel, P. J. Blackshear, and J. C. Lawrence, Jr.** 1995. Control of PHAS-I by insulin in 3T3-L1 adipocytes. J. Biol. Chem. **270:**18531–18538.
42. **Mader, S., H. Lee, A. Pause, and N. Sonenberg.** 1995. The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4γ and the translational repressors 4E-binding proteins. Mol. Cell. Biol. **15:**4990–4997.
43. **Manzella, J. M., W. Rychlik, R. E. Rhoads, J. W. B. Hershey, and P. J. Blackshear.** 1991. Insulin induction of ornithine decarboxylase. J. Biol. Chem. **266:**2383–2389.
44. **Meloche, S., G. Pagès, and J. Pouysségur.** 1992. Functional expression and growth factor activation of an epitope-tagged p44 mitogen-activated protein kinase, p44^{mapk}. Mol. Biol. Cell **3:**63–71.
45. **Mendez, R., M. G. Myers, Jr., M. F. White, and R. E. Rhoads.** 1996. Stimulation of protein synthesis, eukaryotic translation initiation factor 4E phosphorylation, and PHAS-I phosphorylation by insulin requires insulin receptor substrate 1 and phosphatidylinositol 3-kinase. Mol. Cell. Biol. **16:**2857–2864.

46. **Meyuhas, O., D. Avni, and S. Shama.** 1996. Translational control of ribosomal protein mRNAs in eukaryotes, p. 363–388. *In* J. W. B. Hershey, M. B. Mathews, and N. Sonenberg (ed.), Translational control. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
47. **Ming, X. F., B. M. T. Burgering, S. Wennström, L. Claesson-Welsh, C. H. Heldin, J. L. Bos, S. C. Kozma, and G. Thomas.** 1994. Activation of p70/p85 S6 kinase by a pathway independent of $p21^{ras}$. Nature **371:**426–429.
48. **Morley, S. J., and G. Thomas.** 1993. Intracellular messengers and the control of protein synthesis, p. 447–483. *In* C. W. Taylor (ed.), The international encyclopedia of pharmacology and therapeutics: intracellular messengers. Pergamon Press, New York, N.Y.
49. **Nairn, A. C., and H. C. Palfrey.** 1987. Identification of the major Mr 100,000 substrate for calmodulin-dependent protein kinase III in mammalian cells as elongation factor-2. J. Biol. Chem. **262:**17299–17303.
50. **Nourse, J., E. Firpo, W. M. Flanagan, S. Coats, K. Polyak, M.-H. Lee, J. Massague, G. R. Crabtree, and J. M. Roberts.** 1994. Interleukin-2-mediated elimination of the $p27^{Kip}$ cyclin-dependent kinase inhibitor prevented by rapamycin. Nature **372:**570–573.
51. **Nygard, O., and L. Nilsson.** 1990. Translational dynamics. Interactions between the translational factors, tRNA and ribosomes during eukaryotic protein synthesis. Eur. J. Biochem. **191:**1–17.
52. **Okayama, H., and C. A. Chen.** 1988. Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. BioTechniques **6:**632–638.
53. **Pause, A., G. J. Belsham, A.-C. Gingras, O. Donzé, T. A. Lin, J. C. Lawrence, Jr., and N. Sonenberg.** 1994. Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5′-cap function. Nature **371:**762–767.
54. **Pearson, R. B., P. B. Dennis, J. W. Han, N. A. Williamson, S. C. Kozma, R. E. H. Wettenhall, and G. Thomas.** 1995. The principal target of rapamycin-induced $p70^{s6k}$ inactivation is a novel phosphorylation site within a conserved hydrophobic domain. EMBO J. **14:**5279–5287.
55. **Price, D. J., J. R. Grove, V. Calvo, J. Avruch, and B. E. Bierer.** 1992. Rapamycin-induced inhibition of the 70-kilodalton S6 protein kinase. Science **257:**973–977.
56. **Proud, C. G.** 1994. Turned on by insulin. Nature **371:**747–748.
57. **Redpath, N. T., E. J. Foulstone, and C. G. Proud.** 1996. Regulation of translation elongation factor-2 by insulin via a rapamycin-sensitive signalling pathway. EMBO J. **15:**2291–2297.
58. **Reinhard, C., A. Fernandez, N. J. C. Lamb, and G. Thomas.** 1994. Nuclear localization of $p85^{s6k}$: functional requirement for entry into S phase. EMBO J. **13:**1557–1565.
59. **Rosenwald, I. B., R. Kaspar, D. Rousseau, L. Gehrke, P. Leboulch, J. J. Chenn, E. V. Schmidt, N. Sonenberg, and I. M. London.** 1995. Eukaryotic translation initiation factor 4E regulates expression of cyclin D1 at transcriptional and post-transcriptional levels. J. Biol. Chem. **270:**21176–21180.
60. **Sabers, C. J., M. M. Martin, G. J. Brunn, J. M. Williams, F. J. Dumont, G. Weiderrecht, and R. T. Abraham.** 1995. Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. J. Biol. Chem. **270:**815–822.
61. **Sanchez, I., R. T. Hughes, B. J. Mayer, K. Yee, J. R. Woodgett, J. Avruch, J. M. Kyriakis, and L. I. Zon.** 1994. Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. Nature **372:**794–798.
62. **Shama, S., D. Avni, R. M. Frederickson, N. Sonenberg, and O. Meyuhas.** 1995. Overexpression of initiation factor eIF-4E does not relieve the translational repression of ribosomal protein mRNAs in quiescent cells. Gene Expr. **4:**241–252.
63. **Shantz, L. M., and A. E. Pegg.** 1994. Overproduction of ornithine decarboxylase caused by relief of translational repression is associated with neoplastic transformation. Cancer Res. **54:**2313–2316.
64. **Siegmann, M., and G. Thomas.** 1987. Separation of multiple phosphorylated forms of 40S ribosomal protein S6 by two dimensional polyacrylamide gel electrophoresis. Methods Enzymol. **146:**362–369.
65. **Sonenberg, N.** 1996. mRNA 5′ cap-binding protein eIF4E and control of cell growth, p. 245–269. *In* J. W. B. Hershey, M. B. Mathews, and N. Sonenberg (ed.), Translational control. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
66. **Sun, X. J., P. Rothenberg, C. R. Kahn, J. M. Backer, E. Araki, P. A. Wilden, D. A. Cahill, B. J. Goldstein, and M. F. White.** 1991. Structure of the insulin receptor substrate IRS-1 defines a unique signal tranduction protein. Nature **352:**73–77.
67. **Sutherland, C., D. G. Campbell, and P. Cohen.** 1993. Identification of insulin stimulated protein kinase-1 as the rabbit equivalent of rskmo-2. Identification of two threonines phosphorylated during activation of mitogen-activated protein kinase. Eur. J. Biochem. **212:**581–588.
68. **Von Manteuffel, S. R., A.-C. Gingras, X.-F. Ming, N. Sonenberg, and G. Thomas.** 1996. 4E-BP1 phosphorylation is mediated by the FRAP-$p70^{s6k}$ pathway and is independent of mitogen-activated protein kinase. Proc. Natl. Acad. Sci. USA **93:**4076–4080.
69. **Weng, Q.-P., K. Andrabi, M. T. Kozlowski, J. R. Grove, and J. Avruch.** 1995. Multiple independent inputs are required for activation of the p70 S6 kinase. Mol. Cell. Biol. **15:**2333–2340.
70. **Whalen, S. G., A.-C. Gingras, L. Amankwa, S. Mader, P. E. Branton, R. Aebersold, and N. Sonenberg.** 1996. Phosphorylation of eIF4E on serine 209 by protein kinase C is inhibited by the translational repressors, 4E-binding proteins. J. Biol. Chem. **271:**11831–11837.
71. **White, M. F., and C. R. Kahn.** 1994. The insulin signalling system. J. Biol. Chem. **269:**1–4.
72. **Ziemiecki, A., R. G. Müller, F. Xiao-Chang, N. E. Hynes, and S. Kozma.** 1990. Oncogenic activation of the human trk proto-oncogene by recombination with the ribosomal large subunit protein L7a. EMBO J. **9:**191–196.