# Among translational effectors, p70$^{S6k}$ is uniquely sensitive to inhibition by glucocorticoids

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Fundamental cellular processes such as cell differentiation and growth, apoptosis and cellular metabolism are regulated differentially by glucocorticoid hormones in a cell-context-related fashion. However, these basic processes are not governed by isolated signals but are influenced by the integration of both synergistic and antagonistic extracellular and intracellular stimuli. Because glucocorticoids and insulin-like growth factor I (IGF-I) reciprocally modulate growth-regulated processes such as translation initiation, especially in skeletal muscle, a study was undertaken to address the nature of this counter-regulation. Quiescent L6 skeletal myoblasts pretreated for 4 h with the synthetic glucocorticoid dexamethasone exhibited a marked attenuation of IGF-I-induced activation of the ribosomal protein S6 kinase (p70$^{S6k}$). The adverse effects of glucocorticoids on the activity of the endogenous enzyme were due to differential dephosphorylation at discrete residues, suggesting that, physiologically, some but not all phosphorylation sites are subject to mitogenic regulation. Furthermore, the translational repressor eIF4E-binding protein 1 (4E-BP1), which in many circumstances is co-ordinately regulated with p70$^{S6k}$, was dephosphorylated in response to glucocorticoids; however, hyperphosphorylation of the protein after stimulation with IGF-I was refractory to inhibition by glucocorticoids, as was its dissociation from its binding partner, eIF4E. Although both basal and IGF-I-stimulated rates of protein synthesis were modestly affected by glucocorticoids, the synthesis of EF1A, whose mRNA precursor is a prototype for the terminal oligopyrimidine ('TOP') transcript family and whose expression is controlled by the activity of p70$^{S6k}$, was markedly affected. Therefore in this cell system it seems that, despite the mutual control of p70$^{S6k}$ and 4E-BP1 that is often observed, p70$^{S6k}$ is more sensitive to down-regulation by glucocorticoids under growth-promoting conditions than is 4E-BP1.

Key words: eIF4E-binding protein 1, elongation factor 1A, protein kinase B.

## INTRODUCTION

Steroid hormones exert highly disparate, yet often profound, influences on fundamental cellular processes such as growth and differentiation, programmed cell death, and metabolism in a manner that is determined by the target cellular background, i.e. cell type and differentiation state. In some cell types, glucocorticoids exert irreversible cytotoxic effects through the induction of apoptotic programmes [1–4], whereas in others, glucocorticoid receptor activation exerts a cytostatic effect characterized by an arrest of the cell cycle in $G_1$; however, this arrest is alleviated on the removal of hormone [5,6]. The cytostatic properties of glucocorticoids are, in part, attributable to the down-regulation of several factors involved in growth promotion such as c-Myc, cyclin D3 and cyclin-dependent kinases 4 [7] and 2. In contrast, the up-regulation of anti-mitogenic effectors such as CCAAT/enhancer binding protein $\alpha$ [8] and cyclin-dependent kinase inhibitors, p21$^{Cip}$ [9,10] and p27$^{Kip}$ [11], which collectively interfere with cell cycle progression, is a consequence of glucocorticoid action. Thus glucocorticoids function anti-mitogenically in particular cell contexts by the initiation of comprehensive genomic programmes that impede multiple growth-promoting pathways.

The up-regulation of protein synthesis and the protein synthetic machinery is a prelude to mitosis and is a mechanism by which overall cell mass is conserved during division (reviewed in [12]). The initiation of mRNA translation is an important point of regulation for overall protein synthesis; its contribution to growth control is underscored by several compelling observations. First, recessive mutations in *cdc33*, the *Saccharomyces cerevisiae* homologue of eIF4E, abrogate cell cycle progression beyond $G_1$ [13]; this effect is reversed by the ectopic expression of Cln3p, the yeast homologue of cyclin 3, implicating eIF4E in the regulation of the expression of cyclin 3, and thus efficient cell cycle entry [14]. In several cell types, eIF4E is reportedly the least abundant initiation factor [15]; its expression is enhanced in cells exposed to mitogenic stimuli [16]. Moreover, the expression of eIF4E has been found to be augmented in various cancers [17–19] and its overexpression is sufficient to transform normal NIH 3T3 and Chinese hamster ovary cells [15]. In contrast, the overexpression of eIF4E-binding proteins 1 and 2 (4E-BP1 and 4E-BP2) partly reverses the transformed phenotype in cells rendered oncogenic by the overexpression of eIF4E [20]. The efficacy of 4E-BPs as translational suppressors is modulated physiologically by phosphorylation: hypophosphorylated forms associate tightly with eIF4E; in doing so they sequester eIF4E from components of the translational machinery necessary for the initiation of cap-mediated translation.

Expression of the translational machinery itself seems subject to mitogenic regulation primarily via the serine/threonine protein

Abbreviations used: 4E-BP1, eIF4E-binding protein 1; ECL, enhanced chemiluminescence; IGF-I, insulin-like growth factor I; p70$^{S6k}$, 70 kDa ribosomal protein S6 kinase; PDK1 and PDK2, phosphoinositide-dependent kinases 1 and 2; PKB, protein kinase B; mTOR, mammalian target of rapamycin; TOP, terminal oligopyrimidine.

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kinase, 70 kDa ribosomal protein S6 kinase ($p70^{S6k}$). The phosphorylation of S6, an integral component of the 40 S ribosomal subunit, by $p70^{S6k}$ preferentially selects those mRNA species bearing a *cis*-acting terminal oligopyrimidine (TOP) motif, for translation. Transcripts encoding ribosomal proteins and translation factors possess this regulatory element and are therefore subject to $p70^{S6k}$-mediated translational regulation. In dividing cells, $p70^{S6k}$ inactivation hinders cell growth [21] and concomitantly attenuates the translational up-regulation of TOP-containing mRNA species [22–24]. Although $p27^{Kip}$ seems to have a key role in growth inhibition imposed by rapamycin [25], owing to rapamycin's potent inhibition of $p70^{S6k}$, its anti-proliferative properties might in part reflect a decreased synthesis of affected translational components [23]. Furthermore, mice expressing a $p70^{S6k}$ gene rendered null by targeted disruption exhibit various growth defects although they remain viable, probably owing to compensatory up-regulation of a distinct S6 kinase gene encoding a novel and potentially redundant kinase termed S6K2 [26]. Together, these findings emphasize the importance of $p70^{S6k}$ in growth control.

In addition to the numerous pro-mitotic targets, glucocorticoids also negatively affect the translational machinery, particularly through modulation of the respective phosphorylation states of $p70^{S6k}$ and 4E-BP1. These effects, at least in skeletal muscle, do not discriminate between differentiation states of the tissue because both quiescent, undifferentiated myoblasts and terminally differentiated skeletal muscle *in vivo* [27] exhibit glucocorticoid-induced inactivation of $p70^{S6k}$ and eIF4E. Thus the translational machinery might represent an additional important target of anti-mitogenic programmes initiated by glucocorticoid hormones in responsive tissues. We therefore sought to characterize the basic nature of counter-regulation of glucocorticoids on mitogen-induced translational regulation by examining the regulatory component composed of $p70^{S6k}$ and 4E-BP1.

## EXPERIMENTAL

### Materials

Enhanced chemiluminescence (ECL) detection kits, donkey anti-rabbit and sheep anti-mouse horseradish peroxidase-conjugated IgGs were purchased from Amersham Life Sciences. Goat anti-mouse and goat anti-rabbit BioMag magnetic beads were purchased from Perseptive Diagnostics. PVDF membranes were purchased from Bio-Rad. [$^{35}$S]Easytag express protein-labelling mix was acquired from NEN Research Products. Anti-$p70^{S6k}$, anti-(phospho-$Ser^{411}$-$p70^{S6k}$), anti-(phospho-$Thr^{421}$/$Ser^{424}$-$p70^{S6k}$) and anti-(4E-BP1) antibodies were purchased from Santa Cruz Biotechnology. Anti-(protein kinase B) (anti-PKB), anti-(phospho-$Thr^{389}$-$p70^{S6k}$), anti-(phospho-$Thr^{308}$-PKB) and anti-(phospho-$Ser^{473}$-PKB) antibodies were purchased from New England Biolabs. Anti-EF1A antibody was purchased from Upstate Biotechnology. Anti-(phospho-S6) antibody was a gift from Dr Morris Birnbaum (University of Pennsylvania, Philadelphia, PA, U.S.A.). Dexamethasone sodium phosphate and insulin-like growth factor I (IGF-I) were purchased from Sigma.

### Cell culture

L6 myoblasts (A.T.C.C., Manassas, VA, U.S.A.) were seeded in culture dishes 60 or 100 mm in diameter in Dulbecco's modified Eagle's medium supplemented with 10 % (w/v) fetal calf serum (HyClone Labs), 100 i.u./ml benzylpenicillin and 100 $\mu$g/ml streptomycin sulphate. Cells were grown to 70–80 % confluence, then induced to quiescence by replacement of the medium with Dulbecco's modified Eagle's medium devoid of serum and antibiotics for 24 h. Dexamethasone (1 $\mu$M) and/or IGF-I (20 ng/ml) were added to serum-arrested cultures as described in the relevant figure legends. Cells were washed twice with ice-cold PBS and harvested in buffer A [50 mM Tris/HCl (pH 7.4)/1 % (v/v) Nonidet P40/0.25 % deoxycholate/150 mM NaCl/1 mM EDTA/1 mM NaF/0.1 mM PMSF/0.2 mM benzamidine/0.8 $\mu$M leupeptin/1 $\mu$g/ml pepstatin/1 mM $Na_3VO_4$]. Cell lysis was continued by agitation with an orbital rocker for 20 min at 4 °C. The extracts were subsequently cleared of insoluble material by centrifugation at 14000 ***g*** for 20 min at 4 °C.

### Immune complex S6 kinase assays

Cell extracts were prepared by lysis in buffer A devoid of deoxycholate as described above. Cleared extracts were normalized for protein content, then diluted 1:1 with ice-cold PBS. Goat anti-rabbit IgG-conjugated BioMag magnetic beads, which had been preincubated with rabbit anti-$p70^{S6k}$ or rabbit preimmune antiserum, were added to the diluted cell extracts, and incubated on an orbital rocker for 1 h at 4 °C. The resultant immune complexes were isolated by using a magnetic rack and washed twice with buffer B [50 mM Tris/HCl (pH 7.4)/150 mM NaCl/5 mM EDTA/0.1 % (v/v) $\beta$-mercaptoethanol/0.5 % (v/v) Triton X-100/50 mM NaF/50 mM $\beta$-glycerophosphate], twice with buffer C [50 mM Tris/HCl (pH 7.4)/500 mM NaCl/5 mM EDTA/0.1 % (v/v) $\beta$-mercaptoethanol/0.5 % (v/v) Triton X-100/50 mM NaF/50 mM $\beta$-glycerophosphate] and twice with PBS, then resuspended in 40 $\mu$l of kinase assay buffer [20 mM MOPS (pH 7.2)/25 mM $\beta$-glycerophosphate/5 mM EGTA/1 mM $Na_3VO_4$/1 mM dithiothreitol/50 $\mu$M substrate peptide (AKRRRLSSLRA; Upstate Biotechnology)/4 $\mu$M protein kinase C inhibitor peptide (Upstate Biotechnology)/0.4 $\mu$M protein kinase A inhibitor peptide (Upstate Biotechnology)/4 $\mu$M compound R24571 (Upstate Biotechnology)]. Reaction mixtures were preheated to 30 °C and reactions were initiated by the addition of 10 $\mu$l of a $Mg^{2+}$/ATP mixture [75 mM $MgCl_2$/500 $\mu$M ATP/1 $\mu$Ci [$\gamma$-$^{32}$P]ATP (3000 Ci/mmol)]. The reactions were terminated after 10 min by spotting a 25 $\mu$l aliquot of the reaction mixture on P81 phosphocellulose paper. The P81 papers were washed extensively in 0.85 % (v/v) phosphoric acid for the next 24 h and then, finally, once with acetone. The P81 papers were dried and the bound radioactivity was quantified by liquid-scintillation spectrometry. Activity was reported as that in $p70^{S6k}$ immunoprecipitates minus that in rabbit preimmune immunoprecipitates.

### Measurement of protein synthesis

Cells were labelled with 5 $\mu$l of [$^{35}$S]Easytag express protein-labelling mix (11 mCi/ml) for 30 min before harvest. Protein synthesis was assayed by monitoring the incorporation of [$^{35}$S]methionine and [$^{35}$S]cysteine into protein as described elsewhere [28].

### Analysis of Western blots

Protein immunoblots were developed by ECL and quantified by scanning densitometry as described previously [29,30].

### Phosphorylation of $p70^{S6k}$

An aliquot of L6 cell extract was added to an equivalent volume of SDS sample buffer and heated at 100 °C for 7 min, then $p70^{S6k}$

was separated into multiple electrophoretic forms by SDS/PAGE. Separated proteins were then transferred to PVDF membranes, which were subsequently incubated with anti-$p70^{S6k}$ antibody or anti-phosphopeptide antibodies directed against $Thr^{389}$, $Ser^{411}$ or $Thr^{421}/Ser^{424}$. It is generally accepted that the electrophoretic mobility of $p70^{S6k}$ is inversely correlated with its degree of phosphorylation [31]. Because the most highly phosphorylated form, and therefore the species exhibiting the slowest migration, is also the most active form of the protein [32], mobility is often an index of $p70^{S6k}$ activation. However, this form of highly retarded mobility is undetectable under conditions of glucocorticoid exposure. Therefore those electrophoretic species migrating more slowly than the fastest signal, designated here as $p70^{S6k}$-$\alpha$, were quantified densitomerically and expressed as the percentage of the total $p70^{S6k}$ in hyperphosphorylated (relative to $p70^{S6k}$-$\alpha$) forms; the results are therefore indicative of $p70^{S6k}$ phosphorylation/activation.

### Quantification of phosphorylated and unphosphorylated 4E-BP1

Quantification of the phosphorylation state of 4E-BP1 was performed exactly as described elsewhere [30]. In brief, cell lysates were subjected to SDS/PAGE, then immunoblotted with anti-(4E-BP1) antibody. In a manner reminiscent of $p70^{S6k}$, the rate of migration of 4E-BP1 is inversely related to increasing phosphorylation of the protein. Three electrophoretic species, referred to as $\alpha$, $\beta$, and $\gamma$, are readily resolved by SDS/PAGE; the most hyperphosphorylated form was designated $\gamma$; the hypophosphorylated form was designated $\alpha$.

### Quantification of eIF4E and 4E-BP1–eIF4E complexes

Quantification of the respective factors and complexes were performed exactly as outlined previously [30]. eIF4E and 4E-BP1–eIF4E complexes were immunoprecipitated from whole cell lysates by using a mouse monoclonal anti-eIF4E antibody. The immune complexes were isolated by incubation with a goat anti-mouse IgG BioMag magnetic bead slurry. In preparation for incubation with antigen–antibody complexes, the beads were blocked in 0.1 % non-fat dried milk in buffer B for 1 h at 4 °C. The beads were captured by means of a magnetic stand, then washed twice with buffer B and once with buffer C. After incubation of the immune conjugates with magnetic beads, the resultant complexes were eluted in SDS sample buffer, then boiled for 5 min. The beads were pelleted by centrifugation; the supernatants were collected and subjected to SDS/PAGE. After separation of the proteins electrophoretically, they were transferred to PVDF membranes. The membranes were incubated with a mouse monoclonal anti-eIF4E antibody or an anti-(4E-BP1) antibody overnight at 4 °C. Finally, the blots were developed with an ECL Western blotting kit.

### Synthesis of EF1A

The synthesis of EF1A was performed by measuring the incorporation of [$^{35}$S]methionine/cysteine into the immunoprecipitated protein as described elsewhere [33].

## RESULTS

Although many of the genomic events that ultimately give rise to the responses to glucocorticoids observed in various cell types require several hours or even days, the attenuation of the translational machinery is manifested as rapidly as 2 h (O. J. Shah, unpublished work). Therefore the acute effects of glucocorticoids on translational regulation were studied after 4 h of administration of dexamethasone. IGF-I is a potent mitogen for cultured L6 myoblasts at physiological concentrations and induced a robust activation of $p70^{S6k}$ activity (Figure 1) that was maximal after 30 min of incubation then gradually declined over the next 90 min. Pretreatment of myoblasts with dexamethasone for 4 h markedly inhibited enzyme activity over the full period of activation. It has long been understood that $p70^{S6k}$ activation is associated with a retardation of electrophoretic mobility on SDS/polyacrylamide gels, presumably owing to the increased incorporation of phosphate into the protein that occurs during activation. Under most conditions, four electrophoretic species are readily discernible after enzyme activation and thus the appearance of slower electrophoretic forms is often used as an index of both phosphorylation and activation. However, the relationship between electrophoretic mobility and activity, although direct, might not always be proportional (compare Figures 2A and 2B). When hyperphosphorylated species (i.e. non-$\alpha$ species) were expressed as a percentage of total enzyme, little difference in the amount of slower electrophoretic forms was apparent in IGF-I-stimulated cells pretreated with glucocorticoids in comparison with IGF-I stimulation alone (Figure 2A). However, closer scrutiny reveals that the signal strength derived from the uppermost band was appreciably less in cell extracts obtained from glucocorticoid treated cells in comparison with those stimulated with IGF-I alone. The disappearance of this signal was physiologically significant because only those electrophoretic forms of slowest mobility are phosphorylated at $Thr^{389}$ [32] (Figure 3A), a residue whose phosphorylation is critical for enzyme activation. As expected, the inhibition of IGF-I-induced $p70^{S6k}$ activity by glucocorticoids was accompanied by a similar decrease in the amount of phospho-S6 as detected by immunoblot analysis with an anti-(phospho-S6) antibody (Figure 2C).

The activity of $p70^{S6k}$ is believed to be modulated through sequential phosphorylation, initially at sites at the C-terminus (i.e. $Ser^{411}$, $Ser^{418}$, $Thr^{421}$ and $Ser^{424}$) and subsequently within the activation loop (i.e. $Thr^{229}$) and within a distinct hydrophobic region (i.e. $Thr^{389}$). Phosphorylation of $Ser^{371}$ also seems to be indispensable for enzyme activation because mutation of this residue to alanine or glutamic acid renders the enzyme refractory to activation by serum, in part by preventing phosphorylation at $Thr^{389}$. Despite the presence of other mitogen-responsive phosphorylation sites (i.e. $Ser^{17}$, $Ser^{367}$ and $Ser^{404}$), mutational analysis implies that the individual contributions of these sites are relatively minor in the overall activation of the kinase. To understand better the mechanism of glucocorticoid-induced inactivation of endogenous $p70^{S6k}$, antibodies raised against various phosphorylation sites were used to determine which, if any, important regulatory sites were dephosphorylated in response to the steroid. Predictably, glucocorticoids induced the dephosphorylation of $Thr^{389}$ under both basal and IGF-I-stimulated conditions (Figure 3A). Interestingly, Ser/Thr,Pro sites at the C-terminus were differentially dephosphorylated in response to glucocorticoids in that phosphorylation was decreased at $Thr^{421}$ and $Ser^{424}$ (Figure 3B), whereas the phosphorylation of $Ser^{411}$ persisted (Figure 3C). Although these three phosphorylation sites lie within reasonable proximity and are similar in context (i.e. all are followed immediately by a proline residue) two proline residues that lie between $Ser^{411}$ and $Thr^{421}$ might sufficiently contort the secondary structure of the kinase and, in doing so, render $Ser^{411}$ and $Thr^{421}$ on opposite sides of the protein. Such a circumstance could confer mutually exclusive

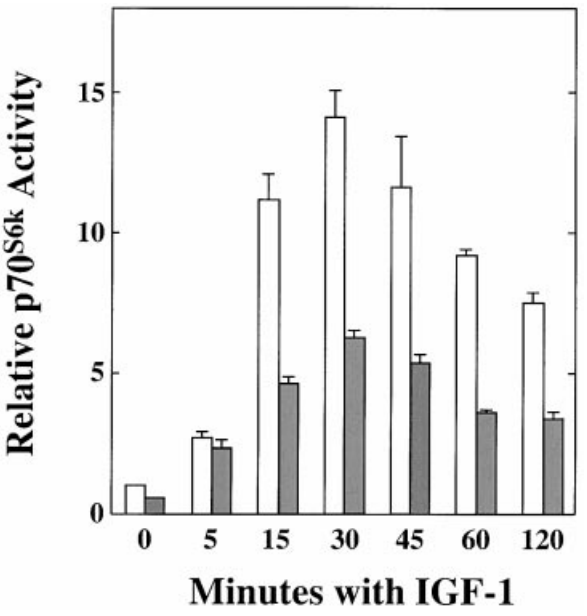


**Figure 1 Glucocorticoids impair the activation of p70$^{S6k}$ by IGF-I**

Serum-arrested skeletal L6 myoblasts were pretreated (grey columns) or not (open columns) with dexamethasone (1 $\mu$M) for 4 h and then with IGF-1 (20 ng/ml) for the additional durations as indicated. Cells were harvested and lysed in preparation for the isolation of p70$^{S6k}$ immune complexes. The immune complexes were then assayed for S6 kinase activity as described in the Experimental section. The results shown are typical of at least three independent experiments, with three dishes per condition. Results are relative kinase activities and are shown as means $\pm$ S.E.M.

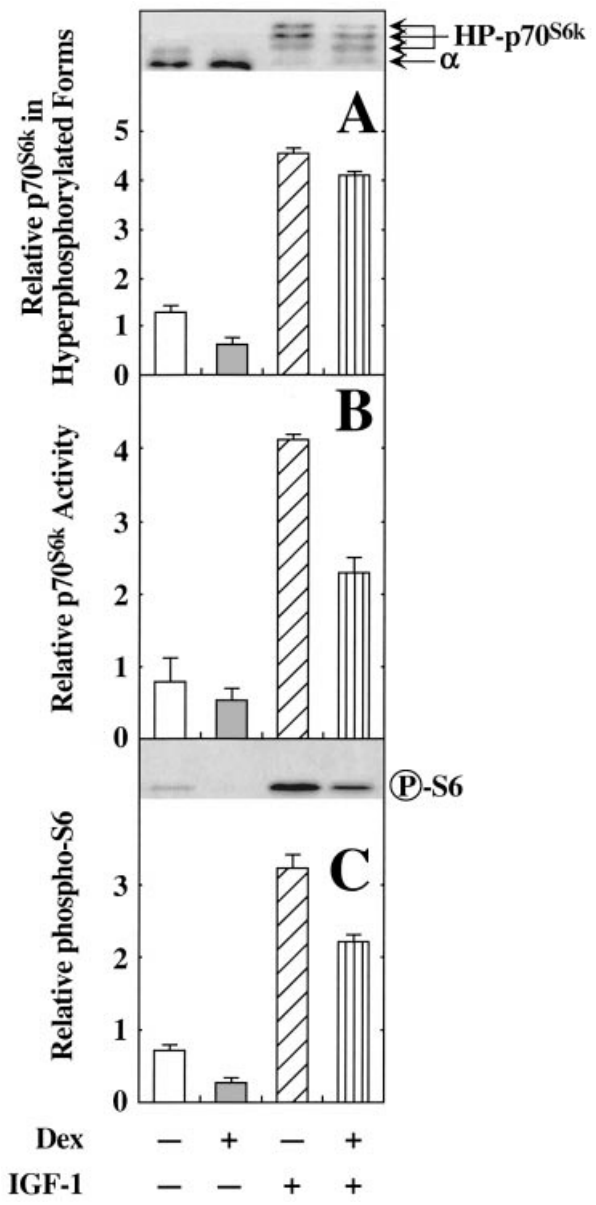


**Figure 2 Relationship between electrophoretic mobility and kinase activity of p70$^{S6k}$ after treatment with glucocorticoid**

Cells were preincubated or not with dexamethasone (Dex) (1 $\mu$M) for 4 h and were then administered IGF-I (20 ng/ml) for a further 30 min. (**A**) Four electrophoretic forms of p70$^{S6k}$ were resolved by SDS/PAGE, then immunoblotted with anti-p70$^{S6k}$ antibody. The relative positions of each species are indicated by arrows and are defined as hypophosphorylated ($\alpha$) or hyperphosphorylated (HP) forms. Each electrophoretic form was quantified and expressed as the ratio of the three HP forms to total p70$^{S6k}$, in arbitrary units. (**B**) S6 kinase assays were performed as described in the legend to Figure 1. (**C**) Proteins derived from cell extracts were separated by SDS/PAGE and immunoblotted with anti-(phospho-S6) antibody. The signals were quantified and expressed in arbitrary units. The results are typical of at least three independent experiments, with three dishes per condition.

regulation of Ser$^{411}$ and Thr$^{421}$/Ser$^{424}$ despite their similarities in context.

The phosphoinositide-dependent kinases (PDKs) 1 and 2 are responsible for the phosphorylation of Thr$^{229}$ [34] and Thr$^{389}$ respectively in p70$^{S6k}$ and of analogous sites in other AGC kinases such as serum- and glucocorticoid-inducible kinase [35] and PKB [36] ('AGC kinases' refers to a family of kinases including protein kinases A, G and C). Although the activity of PDK1 is believed to be constitutive, its phosphoinositide-mediated co-localization at the membrane with cellular targets is believed to impart its regulation and associated activation [37]. In contrast, 'PDK2' is a provisional designation for an unidentified kinase activity believed to function synergistically with PDK1 in the activation of relevant targets. To address the potential of glucocorticoids to down-regulate PDK1 and PDK2 activities, the phosphorylation states of Thr$^{308}$ and Ser$^{473}$ within PKB were examined in the presence of IGF-I and/or glucocorticoids. These sites are analogous to Thr$^{229}$ and Thr$^{389}$ respectively in p70$^{S6k}$ (reviewed in [38]) and are therefore substrates for PDK1 and PDK2 respectively; thus the phosphorylation states of these sites serve as reasonable indices of PDK1 and PDK2 activation. Whereas IGF-I-stimulated phosphorylation of Thr$^{308}$ was augmented by 27% by glucocorticoid pretreatment, phospho-Ser$^{473}$ increased by 65% (Figure 4), suggesting not simply that PDK1- and PDK2-mediated regulation of a physiological substrate remains functional but also that circumstances that promote PKB phosphorylation at both sites are favoured in glucocorticoid-treated cells. Furthermore, because IGF-induced PKB phosphorylation is undiminished in response to glucocorticoids, the likelihood that glucocorticoids induce p70$^{S6k}$ inhibition by down-regulating the activity of phosphoinositide 3-kinase is remote. In fact, in CTLL-20 cells, IL-2-induced phosphoinositide 3-kinase activity is unaffected by glucocorticoids despite a potent attenuation of p70$^{S6k}$ activity by the steroid [39]. Importantly, because PDK1- and PDK2-regulated targets remain at least equally responsive to mitogenic stimuli despite pretreatment with glucocorticoids, direct inac-

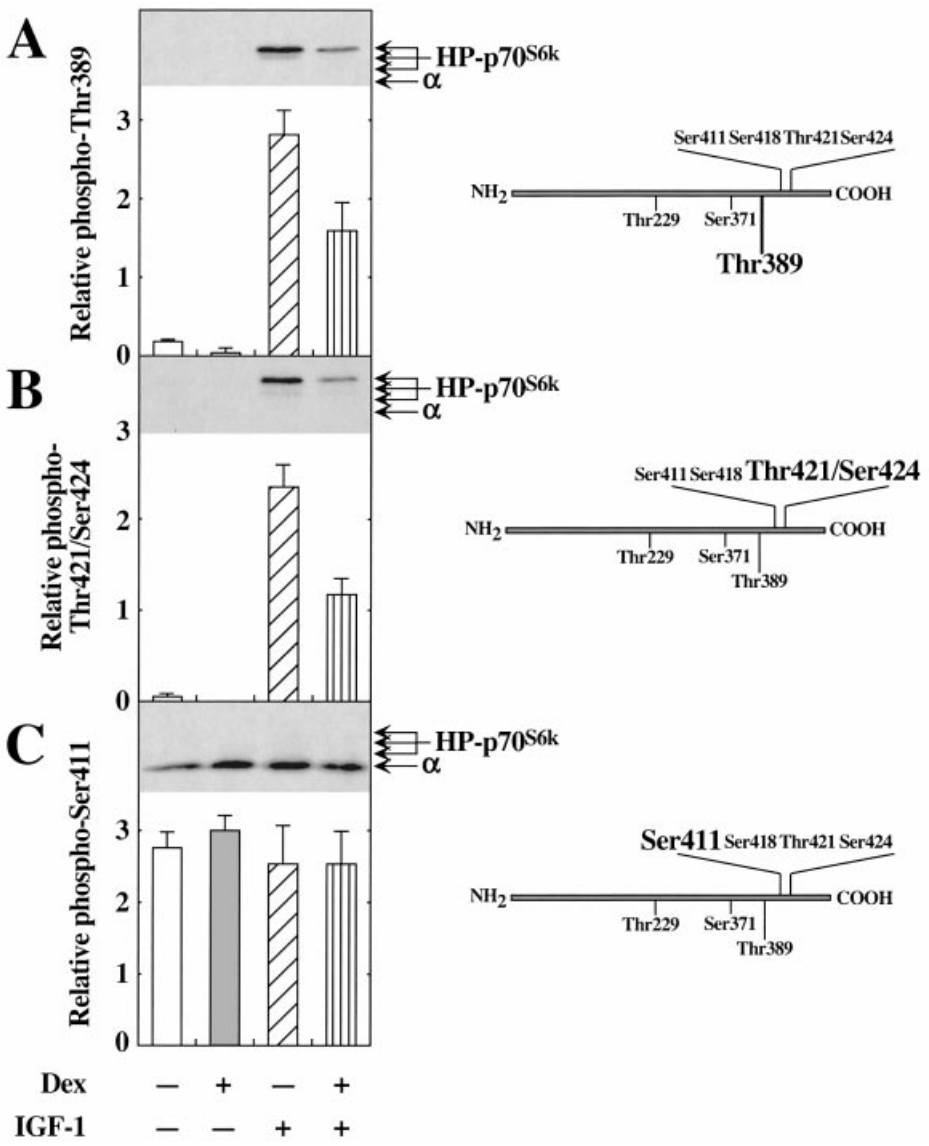


**Figure 3 Inactivation of p70$^{S6k}$ by glucocorticoids is associated with differential dephosphorylation of the enzyme at distinct sites**

Cells were treated as described in the legend to Figure 2; cell extracts were then prepared for Western blot analysis. The electrophoretic forms of p70$^{S6k}$ were resolved by SDS/PAGE and then immunoblotted with phospho-specific antibodies directed against Thr$^{389}$ (**A**), Thr$^{421}$/Ser$^{424}$ (**B**) or Ser$^{411}$ (**C**). The signals were quantified, and means ± S.E.M. expressed, in arbitrary units. After the initial Western analysis, the immunoblots were stripped and reprobed with an anti-p70$^{S6k}$ antibody that recognized all electrophoretic species to determine the relative positions of each of the enzyme's phosphorylated forms. The relative positions of each species are indicated by arrows and are defined as hypophosphorylated ($\alpha$) or hyperphosphorylated (HP) forms. The relative positions of phoshporylation sites are depicted schematically in the right panels. The results are representative of at least five independent experiments, with three dishes per condition, and are means ± S.E.M. Abbreviation: Dex, dexamethasone.

tivation of PDK1 and/or PDK2 by glucocorticoids is unlikely to account for the inactivation of p70$^{S6k}$ in this model.

In many physiological circumstances, the phosphorylation states of p70$^{S6k}$ and 4E-BP1 seem to be co-ordinately regulated, depending on the nature of the stimulus. Reminiscent of p70$^{S6k}$, the electrophoretic mobility of 4E-BP1 is retarded by phosphorylation such that three species designated $\alpha$, $\beta$ and $\gamma$ are resolved on SDS-polyacrylamide gels; the $\gamma$ (hyperphosphorylated) form of the protein does not associate with eIF4E, whereas the $\alpha$ (hypophosphorylated) form, as well as 4E-BP1-$\beta$, readily couples with eIF4E. In quiescent cells, glucocorticoids induced the dephosphorylation of 4E-BP1 (Figure 5A), an event that facilitated its physical association with eIF4E (Figure 5B). However, at doses of dexamethasone that robustly inhibited IGF-I-induced p70$^{S6k}$ activation, the relative proportion of 4E-BP1-$\gamma$ was largely unchanged, as was the coupling of 4E-BP1 to eIF4E (Figures 5A and 5B). Because increased phosphorylation of eIF4E enhances its association with the 5′-methylguanosine cap of mRNA, it has been proposed that eIF4E phosphorylation facilitates the initiation of translation. However, although glucocorticoids do not affect the phosphorylation state of eIF4E in this system, the stimulation of myoblasts with IGF-I induces a redistribution of total eIF4E into dephosphorylated forms. Therefore, despite numerous reports that phospho-eIF4E

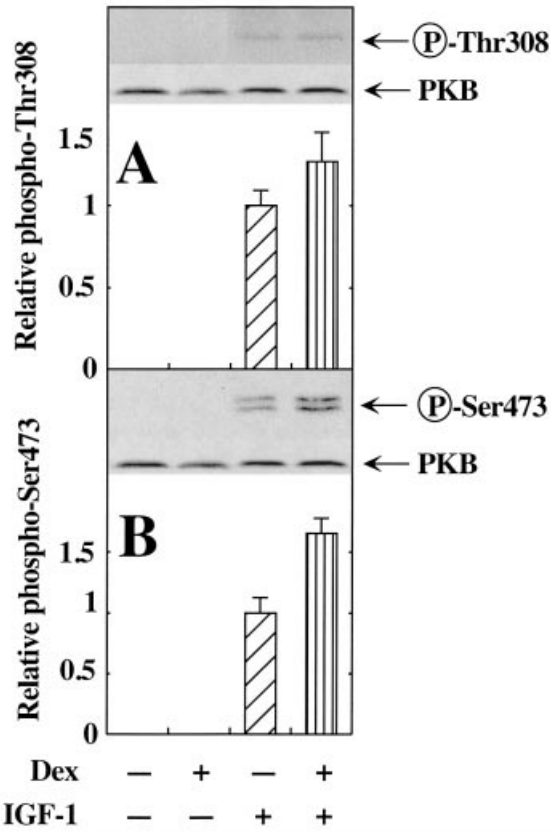


**Figure 4 Phosphorylation of PDK1 and PDK2 substrates, Thr$^{308}$ and Ser$^{473}$ respectively, in PKB is undiminished by glucocorticoids**

Cells were treated as described in the legend to Figure 2. Cell extracts were subjected to SDS/PAGE and then immunoblotted with anti-(phospho-Thr$^{308}$) (**A**), anti-(phospho-Ser$^{473}$) (**B**) or anti-PKB (**A**, **B**) antibodies. The signals were quantified and expressed as the ratio of the respective phosphorylation signal to total PKB, in arbitrary units. Results are means ± S.E.M. and are representative of at least five experiments, with three dishes per condition. Abbreviation: Dex, dexamethasone.

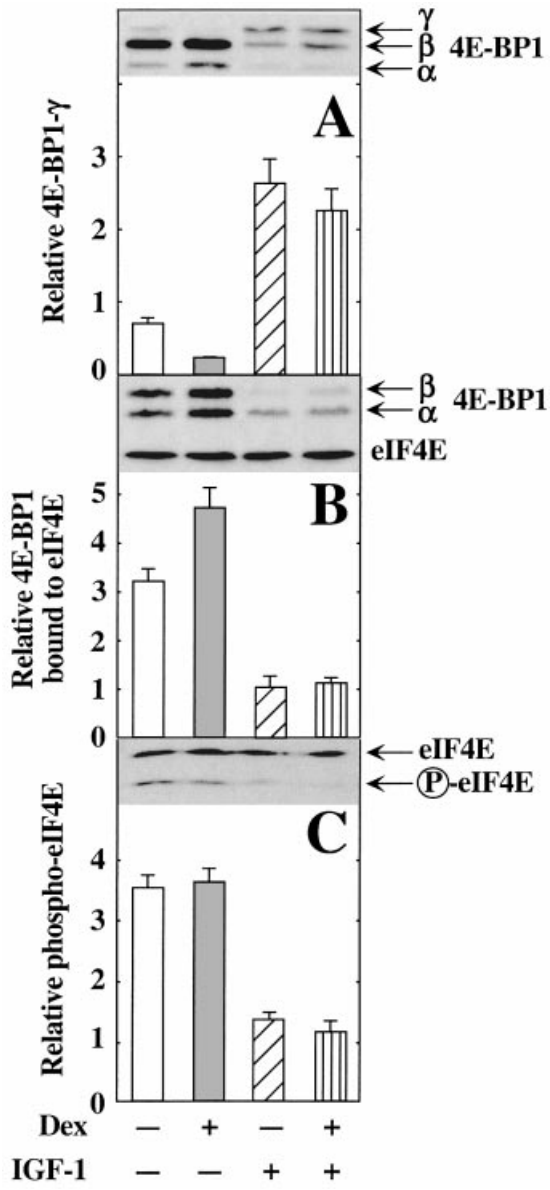


**Figure 5 Effect of glucocorticoids of eIF4E--4E-BP1 complexes in quiescent and IGF-I-stimulated cells**

Cells were treated as described in the legend to Figure 2. (**A**) Electrophoretic species of 4E-BP1 were resolved by SDS/PAGE and then immunoblotted with anti-(4E-BP1) antibody. The signals from each electrophoretic form were quantified and expressed as the ratio of 4E-BP1-$\gamma$ to total 4E-BP1, in arbitrary units. (**B**) eIF4E was immunoprecipitated from cell extracts; the resulting immunoprecipitates were subjected to SDS/PAGE as described in the Experimental section. Only 4E-BP1-$\alpha$ and 4E-BP1-$\beta$ co-immunoprecipitated with eIF4E; these are indicated by arrows. The association of eIF4E with 4E-BP1 is expressed as the ratio of 4E-BP1 to eIF4E in eIF4E immunoprecipitates. (**C**) Cell extracts were prepared and phospho-eIF4E was separated from its dephosphorylated counterpart by slab-gel isoelectric focusing as described in the Experimental section. Results are means ± S.E.M. and are representative of at least three independent experiments, with three dishes per condition. Abbreviation: Dex, dexamethasone.

is preferred under conditions of growth promotion (reviewed in [40]), such phenomena are not without exception.

Translation initiation and, particularly, cap-mediated translation initiation are events believed to govern protein synthetic rate *in vivo* under physiological conditions. In fully differentiated skeletal muscle, glucocorticoids inhibit protein synthetic rates by 20–30 % depending on muscle type [27]. We therefore sought to determine whether the overall protein synthetic response to glucocorticoids could be recapitulated in the undifferentiated myoblast progenitor. Glucocorticoids decreased the rate of protein synthesis in serum-deprived and IGF-I-stimulated cells by 9 % and 6 % respectively relative to the appropriate control (Figure 6A). These changes are, as expected, in parallel with the changes in the phosphorylation state of 4E-BP1 and its coupling to eIF4E (compare Figures 6A, 5A and 5B).

The enzymic activity of p70$^{S6k}$ controls the phospho-S6-mediated translational selection of a unique family of mRNA species whose defining characteristic is a pyrimidine-rich nucleotide signature within their 5′ untranslated region. Because glucocorticoids exert marked effects on the activity of p70$^{S6k}$, we sought to determine whether or not the down-regulation of p70$^{S6k}$ was associated with diminished synthesis of EF1A, whose cognate mRNA is a prototypical TOP-bearing transcript and whose expression is regulated translationally through the activation of p70$^{S6k}$. Indeed, the synthesis of EF1A was decreased by approx. 30 % in quiescent cells administered with glucocorticoids, whereas, in contrast with most cellular proteins (Figure 6A), the IGF-I-induced synthesis of EF1A was abrogated by steroid pretreatment (Figure 6B).

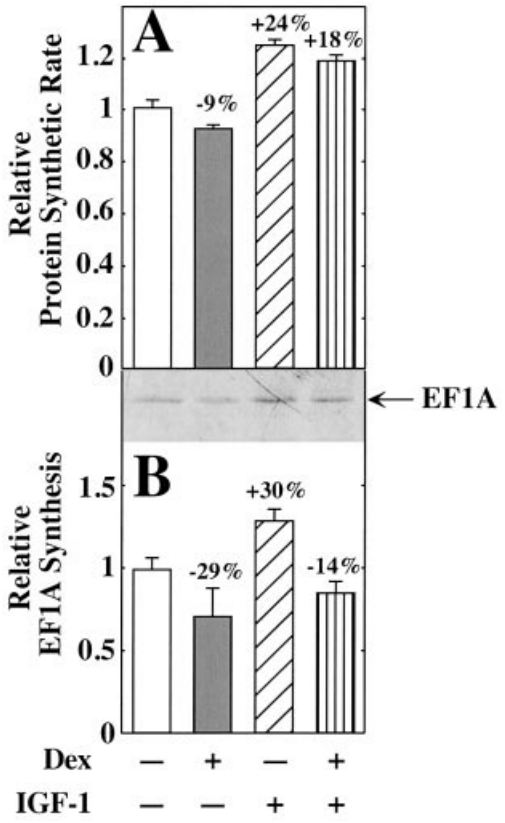


**Figure 6 Glucocorticoids abrogate IGF-I-induced translational selection of a TOP-bearing mRNA**

Cells were treated with dexamethasone (Dex) (1 $\mu$M) for 4 h and then with IGF-I (20 ng/ml) for a further 1 h. During the final 30 min of incubation with IGF-I, a [$^{35}$S]Met/[$^{35}$S]Cys labelling mixture was added to the cultures. (**A**) Total protein from cell extracts was precipitated on filter papers; radioactivity adhering to the filters was determined by liquid-scintillation spectrometry as described in the Experimental section. The results are expressed in arbitrary units; percentage changes from those in untreated cells are shown. (**B**) EF1A was immunoprecipitated from radiolabelled cell extracts and subjected to SDS/PAGE. The proteins in EF1A immunoprecipitates were transferred to PVDF membranes; the rate of synthesis was estimated by quantification of the autoradiographic signal derived from EF1A as described in the Experimental section. The identity of the signal was confirmed by subsequent Western blotting with anti-EF1A antibody. The results are expressed as arbitrary units and are means $\pm$ S.E.M. for 12 dishes per condition.

## DISCUSSION

The importance of p70$^{S6k}$ in growth control is highlighted by several intriguing observations. First, the microinjection of neutralizing anti-p70$^{S6k}$ antibodies induces an arrest of the cell cycle in $G_1$ similar to that observed in cells treated with rapamycin, underscoring the necessity of p70$^{S6k}$ activity for efficient cell cycle transit. The activity of p70$^{S6k}$ is cyclic: it is elevated at both the $G_0/G_1$ and the $M/G_1$ boundaries, diminishes precipitously throughout the remainder of the cell cycle and ultimately rises again with the entrance into the next cycle [41]. Moreover, p70$^{S6k}$ activity seems to be required for DNA synthesis [42] and for the regulation of growth-related processes such as E2F transcriptional competence [43], and is a critical determinant of cell size in *Drosophila* [44].

This investigation has revealed that, whereas glucocorticoids down-regulate p70$^{S6k}$ and eIF4E function in quiescent cells, only p70$^{S6k}$ remains sensitive to glucocorticoids after stimulation with mitogen. Accordingly, the relative changes in general protein synthesis after treatment with glucocorticoid are mild compared with the steroid-induced inhibition of IGF-I-stimulated EF1A synthesis, suggesting that glucocorticoids have a significant role in the regulation of TOP mRNA translation. The mitogen-induced translation of this mRNA family is abrogated in mammalian cells pretreated with rapamycin [24], which also potently inactivates p70$^{S6k}$. Rapamycin-sensitive translation is conferred on chimaeric mRNA species bearing wild-type, but not mutant, TOP sequences [24]. Furthermore, the expression of mRNA species bearing the wild-type, but not disrupted, sequences is inhibited by an ectopically expressed dominant-negative p70$^{S6k}$ variant [24], demonstrating that p70$^{S6k}$ activity is necessary for the mitogen-stimulated translational up-regulation of TOP-containing transcripts. Rapamycin also selectively suppresses the translation of elongation factor and ribosomal protein mRNA species without affecting the synthesis of non-ribosomal proteins [22]. Thus the inhibition of IGF-I-induced EF1A translation by glucocorticoids probably derives from the associated down-regulation of p70$^{S6k}$ activity.

The mechanism by which glucocorticoids attenuate p70$^{S6k}$ activation is incompletely understood but seems to require a functional glucocorticoid receptor because both the glucocorticoid receptor antagonist mifepristone (RU486) and the heat shock protein 90 inhibitor geldanamycin partly protect p70$^{S6k}$ from inactivation by glucocorticoids (O. J. Shah, unpublished work). Furthermore, the down-regulation of p70$^{S6k}$ and eIF4E in serum-deprived cells requires a temporal lag of at least 2 h and is sensitive to translational and transcriptional inhibition, suggesting a transcriptional requirement (i.e. activation and/or repression) for the regulation of p70$^{S6k}$ and eIF4E by glucocorticoids. Although the glucocorticoid receptor is capable of both induction and repression via direct physical contact with target elements upstream of responsive genes, the receptor can also interfere with other transcriptional pathways through DNA-independent protein–protein interactions, of which cross-talk with nuclear factor $\kappa$B [45,46] and activator protein 1 [47] are classical examples. Indeed, the potential of the glucocorticoid receptor as a DNA-independent transcriptional modulator has received heightened interest as knock-in mice expressing a dimerization-deficient (and putatively DNA-binding-deficient) glucocorticoid receptor variant remain viable [48]. As yet the results support no conclusions on the DNA dependence of glucocorticoid-controlled translation. Identification of the transcriptional properties of the glucocorticoid receptor required for the phenomena reported here will certainly serve to illuminate this issue; such a study is currently in progress.

The regulation of p70$^{S6k}$ is mediated by phosphorylation at multiple sites, several of which function independently yet co-operatively with other activating phosphorylation events. Regulation of the enzyme could therefore occur through the inhibition of one or more p70$^{S6k}$ kinases. Alternatively, because the regulation of discrete sites is predicted to occur in an ordered and successive manner, it seems plausible that the inactivation of initial phosphorylation events would render the enzyme refractory to subsequent activating inputs. Phosphorylation of a C-terminal cluster of four Ser/Thr,Pro sites (i.e. Ser$^{411}$, Ser$^{418}$, Thr$^{421}$ and Ser$^{424}$) is believed to prime the kinase for activation by inducing a conformational change that relieves the intramolecular occlusion of the catalytic pocket by the C-terminal pseudosubstrate domain. Mutation of these residues to alanine decreases both basal and mitogen-induced p70$^{S6k}$ activation to 30% of their respective levels, whereas substitutions of acidic residues at these sites enhance basal kinase activity without affecting mitogen-stimulated activity. Moreover, a p70$^{S6k}$ truncation mutant in which the C-terminus (which includes Ser$^{411}$,

$Ser^{418}$, $Thr^{421}$ and $Ser^{424}$ as well as $Ser^{404}$) has been deleted retains 75% of the serum-stimulated activity observed for the wild-type enzyme. Cumulatively, these results suggest that the C-terminus is largely dispensable for enzyme activity but has a key, but indirect, role in regulating enzyme activation by affecting the accessibility of the remaining phosphorylation sites (especially $Thr^{229}$, $Ser^{371}$ and $Thr^{389}$) to $p70^{S6k}$ kinases. Our findings are consistent with such a model in that glucocorticoid-induced inactivation of $p70^{S6k}$ is associated with dephosphorylation of $Ser^{421}/Thr^{424}$ and $Thr^{389}$. Further, because phosphorylation of PKB at $Thr^{308}$ and $Ser^{473}$, sites analogous to $Thr^{229}$ and $Thr^{389}$ in $p70^{S6k}$, is retained in glucocorticoid-treated cells, it is unlikely that glucocorticoid-induced down-regulation of $p70^{S6k}$ is due to the inactivation of $Thr^{229}$ or $Thr^{389}$ kinases (i.e. PDK1 or PDK2 respectively). Therefore the observed dephosphorylation of $Thr^{389}$ might be secondary to the dephosphorylation of $Ser^{421}/Thr^{424}$, which would be predicted to mask internal sites of potential phosphorylation including $Thr^{389}$. Despite these findings, the possibility that glucocorticoids inhibit other $p70^{S6k}$ kinases (e.g. the $Ser^{371}$ kinase) cannot be excluded.

Nevertheless, if glucocorticoids promote the dephosphorylation of the C-terminus, other critical phosphorylation sites (e.g. $Thr^{229}$ and $Ser^{371}$) would be expected to be dephosphorylated. Moreover, a $p70^{S6k}$ variant bearing acidic substitutions at the clustered phosphorylation sites within the pseudosubstrate domain would be expected to exhibit glucocorticoid-resistant S6 kinase activity. Investigations are currently under way to address this issue.

Although $Ser^{411}$ lies within a Ser/Thr,Pro context and is situated adjacent to similar sites (i.e. $Ser^{418}$, $Thr^{421}$ and $Ser^{424}$), several findings suggest that its regulation might indeed be unique. Phosphorylation of $Ser^{411}$ is observed during mitosis, a cell cycle phase characterized by low $p70^{S6k}$ activity [49]. The Cdc2–cyclin B complex is believed to phosphorylate this site and is postulated to contribute to the decreased $p70^{S6k}$ activity detected in mitosis [49]. In serum-arrested L6 myoblasts, neither glucocorticoids nor rapamycin (O. J. Shah, unpublished work) affect the phosphorylation of this residue. Furthermore, its phosphorylation is not enhanced after stimulation with IGF-I (Figure 3C), unlike that of $Thr^{389}$ and $Thr^{421}/Ser^{424}$ (Figures 3A and 3B). $Ser^{411}$ is unique among the C-terminal proline-directed phosphorylation sites in that it lies within a conserved recognition motif for the mitotically regulated peptidyl-prolyl isomerase Pin1 [50]. A physical association has been demonstrated between Pin1 and $p70^{S6k}$ during mitosis but not during interphase [50]. Although the significance of this interaction has not been established, the coupling of Pin1 to $p70^{S6k}$ during a cell cycle phase of low kinase activity together with a requirement for $Ser^{411}$ phosphorylation suggests that this residue might function as a cell cycle-regulated molecular adapter rather than contributing directly to mitogen-induced enzyme activation.

Whereas both 4E-BP1 and $p70^{S6k}$ are rapidly and effectively dephosphorylated in cells administered rapamycin and whereas both effectors are reportedly subject to phosphorylation by the mammalian target of rapamycin (mTOR), the regulations of their respective phosphorylation states and activities are differential under the conditions used in the present study. Because mTOR has been demonstrated to phosphorylate both $p70^{S6k}$ and 4E-BP1, down-modulation of an mTOR-mediated pathway might explain the current observations. Immunopurified mTOR phosphorylates $Thr^{37}$ and $Thr^{46}$ in 4E-BP1; however, phosphorylation of these sites is relatively constitutive and is not sufficient to remove the inhibition from eIF4E [51]. Recently, Heesom and Denton [52] have described an mTOR-precipitable $Ser^{64}$ kinase activity distinct from that activity intrinsic to mTOR and that induces the dissociation of 4E-BP1 from eIF4E. With regard to $p70^{S6k}$, phosphorylation by mTOR *in vitro* occurs with dual context specificity as shown by the efficient phosphorylation of both $Thr^{389}$ (which lies within a hydrophobic amino acid context) and $Thr^{421}/Ser^{424}$ (which each lie within a Ser/Thr,Pro context) [53]. In view of this evidence, it is possible that the regulation of mTOR alone by glucocorticoids could account for the discordant regulation of 4E-BP1 and $p70^{S6k}$ observed in cells stimulated with IGF-I. In such a model, inhibition of mTOR's inherent protein kinase activity would sufficiently deter the phosphorylation of both $Thr^{389}$ and $Thr^{421}/Ser^{424}$ in $p70^{S6k}$, whereas the stability of the eIF4E/4E-BP1 complex would be refractory to the dephosphorylation of $Thr^{37}$ and $Thr^{46}$ (if such dephosphorylation did indeed occur). Although the expression of the mTOR protein is not affected by glucocorticoids within 4 h (O. J. Shah, unpublished work), the influence of these hormones on mTOR's catalytic activity is not known.

The anti-proliferative properties of glucocorticoids derive from complex genomic programmes that cumulatively deter cell growth and division. In view of the intricate co-ordinated control of cell growth and proliferation and the relationship of $G_1$ progression to cellular growth rate, the down-regulation of $p70^{S6k}$ and affected translational components might represent a method by which glucocorticoids simultaneously affect the translational capacity of the cell and its proliferation. The mechanisms by which glucocorticoids modulate $p70^{S6k}$ and the identity of the involved regulator(s) will undoubtedly enhance our understanding of selective translational control and its contribution to growth regulation.

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