

Regulation of targets of mTOR (mammalian target of rapamycin) signalling by intracellular amino acid availability

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In mammalian cells, amino acids affect the phosphorylation state and function of several proteins involved in mRNA translation that are regulated via the rapamycin-sensitive mTOR (mammalian target of rapamycin) pathway. These include ribosomal protein S6 kinase, S6K1, and eukaryotic initiation factor 4E-binding protein, 4E-BP1. Amino acids, especially branched-chain amino acids, such as leucine, promote phosphorylation of 4E-BP1 and S6K1, and permit insulin to further increase their phosphorylation. However, it is not clear whether these effects are exerted by extracellular or intracellular amino acids. Inhibition of protein synthesis is expected to increase the intracellular level of amino acids, whereas inhibiting proteolysis has the opposite effect. We show in the present study that inhibition of protein synthesis by any of several protein synthesis inhibitors tested allows insulin to regulate 4E-BP1 or S6K1 in amino-acid-deprived cells, as

does the addition of amino acids to the medium. In particular, insulin activates S6K1 and promotes initiation factor complex assembly in amino-acid-deprived cells treated with protein synthesis inhibitors, but cannot do so in the absence of these compounds. Their effects occur at concentrations commensurate with their inhibition of protein synthesis and are not due to activation of stress-activated kinase cascades. Inhibition of protein breakdown (autophagy) impairs the ability of insulin to regulate 4E-BP1 or S6K1 under such conditions. These and other data presented in the current study are consistent with the idea that it is intracellular amino acid levels that regulate mTOR signalling.

Key words: cycloheximide, initiation factor, mRNA translation, phosphorylation, S6 kinase.

INTRODUCTION

Recent studies have shown that nutrients, especially amino acids, modulate the activity of a number of proteins involved in the control of mRNA translation (for recent reviews, see [1,2]). Several of them are controlled by phosphorylation through signalling events involving the mammalian target of rapamycin (mTOR) [3,4], the function of which is inhibited potently by the immunosuppressant rapamycin. This has led to the concept that mTOR couples nutrient availability to downstream signalling to translation factors, such that amino acid supply acts as a feed-forward activator of proteins that regulate protein synthesis.

Several components of the translational machinery are regulated by mTOR signalling. These include the translational repressor protein eukaryotic initiation factor (eIF) 4E-binding protein 1 (4E-BP1) [5,6], the ribosomal protein S6 kinases S6K1 and S6K2 [2,7] and elongation factor 2 [4]. 4E-BP1 interacts with eIF4E, the protein that binds to the 5'-cap structure of eukaryotic mRNAs (for review, see [5]). eIF4E also binds eIF4G, a scaffold protein that interacts further with eIF3 and recruits the 40 S ribosome to the 5'-end of the mRNA. eIF4G binds the RNA helicase eIF4A, and this is thought to play a role in facilitating translation of mRNAs which possess significant secondary structure in their 5'-untranslated regions [8]. The complex comprising eIF4A, eIF4E and eIF4G is often termed eIF4F and is considered to be critical for cap-dependent mRNA translation. eIF4G and 4E-BP1 bind to overlapping sites on eIF4E, so their interactions with eIF4E are mutually exclusive

[5]. 4E-BP1 thus represses formation of eIF4F complexes. 4E-BP1 is a phosphoprotein, and stimuli such as insulin increase its phosphorylation, leading to its release from eIF4E and allowing eIF4E to bind eIF4G. Insulin-induced phosphorylation of 4E-BP1 is blocked by rapamycin, thus implicating mTOR in the signalling events upstream of 4E-BP1 [9–11].

Insulin also induces the phosphorylation and activation S6K1 [2,7] and S6K2 [12,13]. This is also blocked by rapamycin. Both kinases undergo phosphorylation at multiple sites in the catalytic domain and in their C-termini. Both phosphorylate ribosomal protein (rp) S6. The function of S6 phosphorylation is not entirely clear: it has been suggested that S6K1, and possibly S6 phosphorylation, positively regulates the translation of a set of mRNAs that are characterized by the presence within their 5'-UTRs of a tract of pyrimidines (TOP). These so-called 5'-TOP mRNAs encode the ribosomal proteins and several other components of the translational machinery in mammals [14]. They are poorly translated in serum-starved cells, but shift into polysomes upon stimulation. This translational up-regulation is sensitive to rapamycin [15] and it was suggested that it was linked to activation of S6K1 [16]. However, recent studies [17,18] have questioned the link between S6K1, S6 phosphorylation and 5'-TOP mRNA translation.

We and others [1,19–21] have shown that the phosphorylation and/or activity of 4E-BP1 and S6K1 are modulated by amino acids in a number of types of cells. For example, whereas insulin regulates these proteins in cells supplied with amino acids, it fails to do so when cells are briefly (approx. 1 h) deprived of

Abbreviations used: 3-MA, 3-methyladenine; 4E-BP1, eIF4E-binding protein 1; BCAA, branched-chain amino acids; CHO, Chinese-hamster ovary; CHX, cycloheximide; D-PBS, Dulbecco's PBS; eIF, eukaryotic initiation factor; GCN2, general control of amino acid biosynthesis, non-derepressing, 2; GSK3, glycogen synthase kinase 3; GST, glutathione S-transferase; hsp, heat-shock protein; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MAPKAPK-2, MAP kinase-activated kinase-2; mTOR, mammalian target of rapamycin; PKB, protein kinase B; rp, ribosomal protein; S6K, rp S6 kinase; TOP, tract of pyrimidines.

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amino acids (reviewed in [1,19]). Resupplying amino acids restores regulation, and leucine was generally found to be the most effective single amino acid, although other branched-chain amino acids (BCAA) may also be effective [1]. In fact, amino acids themselves increase the phosphorylation and activity of S6K1 and 4E-BP1, and can allow formation of eIF4F complexes even in the absence of insulin, at least in some types of cells {e.g. Chinese-hamster ovary (CHO) cells [20,21]}. Glucose seems to provide a permissive input to the control of 4E-BP1 and eEF2 [20,21]. This may be related to cellular ATP levels, as mTOR has been reported to have a high K_m for ATP [22].

The positive regulatory, or permissive, role of amino acids in the control of translation factors can easily be rationalized in terms of their physiological control: in the absence of an adequate supply of amino acids, it makes little physiological sense to activate translation initiation or elongation, or to stimulate overall protein synthesis. Thus amino acids act as permissive or feed-forward regulators of translation factor activity. Within the cell, amino acids may be derived from two main sources: from extracellular amino acids, via their uptake, or from protein degradation, which also provides amino acids for the intracellular pool.

It is unclear how mammalian cells perceive the prevailing amino acid supply and relay this information to signalling pathways within the cell. There are two main possibilities. Firstly, mammalian cells may possess a plasma-membrane amino acid sensor similar in concept to the yeast sensor Ssy1p [23], which detects external amino acid levels. This would presumably be coupled to a signalling cascade or transduction pathway within the cytoplasm of the cell linked to control of mTOR. Early studies from Mortimore and co-workers [24] provided some evidence for a cell-surface receptor for leucine, although this receptor has not been identified. Alternatively, mammalian cells may possess an intracellular amino acid sensor, linked, in an unknown way, to the signalling machinery of the cell and, specifically, to mTOR. Recent data from the amphibian *Xenopus laevis* suggests that its oocytes sense intracellular amino acids [25].

In the present study, we have used a number of approaches to investigate the relationship between amino acid supply and the regulation of targets of mTOR signalling. Our data are consistent with the idea that mammalian cells sense intracellular amino acid pools, the size of which is determined by the extracellular amino acid supply and by rates of protein synthesis and degradation.

MATERIALS AND METHODS

Chemicals and biochemicals

Unless otherwise indicated, all reagents were obtained from Sigma or Merck. Antisera for 4E-BP1, eIF4E, eIF4G, S6K1, phosphorylated eIF2 α , phosphorylated eEF2 and eEF2 were as described previously [20,26–30]. Anti-phospho-S6K1 (Thr³⁸⁹) was from New England Biolabs (Hitchin, Herts., U.K.). Anti-phospho-rpS6 (Ser²³⁵) was from Dr Dario Alessi (University of Dundee; see also [21] for further information). Cycloheximide (CHX), anisomycin and 3-methyladenine (3-MA) were from Sigma. Antibodies to phosphorylated protein kinase B (PKB) or glycogen synthase kinase 3 (GSK3) were from Cell Signaling Technology (Beverly, MA, USA).

Cell culture and treatment

CHO K1 cells were maintained in Ham's F12 medium as described previously [31]. When cells were approx. 80% confluent, they were starved of serum for 4 h by transferring them to the

same medium but without serum. After removal of the medium, some dishes were rinsed once with Dulbecco's phosphate buffered saline (D-PBS) containing 10 mM D-glucose (D-PBS/glucose) and then incubated in the same solution for 1 h, whereas controls remained in Ham's F12 medium. In some cases (where indicated), cells were treated with insulin (100 nM, 10 min). Where added, amino acids were supplied as a mixture. The stock solution contained 5 mM L-arginine, 4 mM L-cysteine, 40 mM L-glutamine, 2.5 mM L-histidine, 8 mM L-isoleucine, 8 mM L-leucine, 10 mM L-lysine, 2 mM L-methionine, 4 mM L-phenylalanine, 8 mM L-threonine, 0.6 mM L-tryptophan, 4 mM L-tyrosine and 8 mM L-valine dissolved in D-PBS/glucose. One-tenth volume of this stock was added to the D-PBS/glucose in which the cells were kept. Unless otherwise indicated, CHX and anisomycin were used at 10 μ g/ml and emetine at 50 μ g/ml, and were added for a further 30 min to cells that had already been kept in D-PBS/glucose for 1 h. Where relevant, insulin was added at the end of this 30 min period. Where used, 3-MA was applied to cells in D-PBS/glucose for 2 h at a concentration of 10 mM. The p38 mitogen-activated protein (MAP) kinase inhibitor SB203580 was used at 10 μ M for 1 h.

Cell lysis and processing of cell lysates

CHO cells were lysed by scraping into standard extraction buffer [50 mM β -glycerophosphate, 1 mM EDTA, 1 mM EGTA, 0.5 mM Na₂VO₄, 1 mM benzamidine hydrochloride, 1 mM dithiothreitol, 1% (v/v) Triton X-100, 0.1 mM PMSF and 1 μ g/ml each of pepstatin, leupeptin and antipain (pH 7.4)]. For analysis of proteins associated with eIF4E, lysates were subjected to affinity chromatography on m⁷GTP-Sepharose as described previously [32]. Protein concentrations were determined by the method of Bradford [33].

Gel electrophoresis and Western blotting

These procedures were performed as described previously [34]. For the analysis of the mobilities of differently phosphorylated forms of 4E-BP1, resolving gels contained 13.5% (w/v) acrylamide and 0.36% (w/v) methylene bisacrylamide were used. For analysis of the association of 4E-BP1 or eIF4G with eIF4E, or to study the phosphorylation of eIF2 α , PKB or GSK3, resolving gels contained 12.5% (w/v) acrylamide and 0.1% (w/v) methylene bisacrylamide. It should be noted that the differentially phosphorylated forms of 4E-BP1 cannot reliably be resolved in the latter gel system. S6K1 was analysed on gels containing 10% (w/v) acrylamide. On each gel, equal amounts of cell protein were applied to each lane and, where possible, equal loading of the samples was confirmed by Western-blot analysis with an appropriate antibody.

Protein kinase assays

The activity of S6K1 was assayed, after immunoprecipitation, using a peptide substrate as described previously [28]. The activation states of p38 MAP kinase α/β were assayed indirectly by using heat-shock protein (hsp) 25 as substrate for their common downstream kinase MAP kinase-activated protein kinase-2 (MAPKAPK-2), as described previously [35]. The activity of the c-Jun N-terminal kinase (JNK) was determined using a glutathione S-transferase (GST) fusion protein containing residues 1–135 of c-Jun [36] to assay the kinase directly in cell lysates.

Amino acid determinations

The concentrations of BCAA were determined by an enzymic fluorimetric assay based on BCAA dehydrogenase, as described previously [37]. Cells were washed and then extracted into perchloric acid. After centrifugation to remove insoluble material, the supernatant (containing the BCAA) was neutralized with KOH and used in the BCAA assay.

Protein synthesis

Protein synthesis was assayed by measuring the incorporation of radiolabelled methionine into trichloroacetic acid-insoluble material. [³⁵S]Methionine (12.5 μ Ci/ml) was added to the cell-culture dish for 30 min. Cells were then lysed in standard extraction buffer. The protein concentrations of the lysates were determined and equal amounts of protein were processed in triplicate for determination of incorporation of radiolabel into trichloroacetic acid-insoluble material, as described previously [38].

Reproducibility

All experiments were performed at least three times, with similar outcomes. In the case of Western blots, data from a typical experiment is shown.

RESULTS

Protein synthesis inhibitors modulate the control of S6K1 and 4E-BP1 in amino-acid-deprived cells

The aim of the studies described in this section was to examine the effect of protein synthesis inhibitors on the phosphorylation, activity and control of targets of mTOR signalling. We have shown previously [20,39] that, when CHO.K1 cells are starved of amino acids, S6K1 and 4E-BP1 undergo dephosphorylation and become unresponsive to insulin. The rationale underlying the experiments described in the present study is as follows: if these effects are due to depletion of intracellular amino acids, it might be expected that inhibiting protein synthesis would maintain the phosphorylation and responsiveness of these proteins by acting to prevent the consumption of amino acids and thus helping maintain, or even increase, the levels of intracellular amino acids, which will continue to be provided by protein breakdown.

CHO.K1 cells were transferred from Ham's F12 medium into D-PBS/glucose, i.e. to a medium that lacks amino acids. Where indicated, cells were also treated with insulin. Lysates were then prepared and samples were analysed by SDS/PAGE and Western blotting using an antiserum that detects S6K1. Differentially phosphorylated forms of the S6K1 differ in their mobilities with the more highly phosphorylated ones migrating more slowly. As judged by this criterion, insulin was unable to elicit the phosphorylation of S6K1 under this condition, whereas insulin did activate S6K1 in cells kept in Ham's F12 medium, which contains amino acids and glucose (Figure 1A). This is consistent with our previous data for similar experiments using CHO cells [20]. Phosphorylation at Thr³⁸⁹ in the extension to the catalytic domain of S6K1 is thought to play a key role in the control of S6K1 via mTOR signalling (reviewed in [2]), and we used a phospho-specific antiserum to study the phosphorylation of this site. It should be noted that this antibody cross-reacts non-specifically with another polypeptide than runs just above S6K1 (indicated by an asterisk in Figure 1A; this serves as a useful loading control). Using the anti-(phospho-Thr³⁸⁹S6K1) antibody,

only a single S6K band was observed, rather than the ladder seen with the phosphorylation-insensitive antibody. This suggests that Thr³⁸⁹ is a 'late' site in the hierarchical phosphorylation of S6K1 that leads to its activation (compare top and middle panels of Figure 1A) [40]. Although insulin increased the phosphorylation of S6K1 at Thr³⁸⁹ in cells kept in Ham's F12 medium, this effect was not observed in cells deprived of amino acids (Figure 1A). Similarly, in cells maintained in Ham's F12 medium, insulin increased the phosphorylation of an intracellular substrate for S6K1, rpS6, but failed to do so in cells deprived of amino acids. To corroborate these data, we also measured the activity of S6K1 following immunoprecipitation by using a standard peptide substrate [28]. As shown in Figure 1(B), cells kept in Ham's F12 medium displayed significant basal S6K1 activity, which was enhanced by insulin, whereas amino-acid-deprived cells showed lower activity and insulin did not increase this. This is consistent with data obtained previously for CHO cell lines overexpressing the insulin receptor [20,39,41].

Addition of CHX to amino-acid-deprived cells, at a concentration which essentially completely inhibited protein synthesis (10 μ g/ml; 92 \pm 3% inhibition), resulted in only a small decrease in the mobility of S6K1 on SDS/PAGE (Figure 1A), giving a similar picture to that seen in cells that were kept in Ham's F12 medium, and had little effect on the basal activity of S6K1 (Figure 1B). The arrowhead in Figure 1(A) indicates a more slowly migrating species of S6K1 that is seen in CHX-treated cells and in cells kept in Ham's F12 medium. However, CHX treatment did permit the phosphorylation and activation of S6K1 in response to insulin (Figures 1A and 1B). CHX also allowed insulin to increase the phosphorylation of S6K1 at Thr³⁸⁹.

This overall picture is very similar to the situation seen in CHO cells kept in Ham's F12 medium, which contains amino acids (see data in Figure 1A), or to the effect of providing external amino acids on the responsiveness of S6K1 to insulin in these cells (see [39] and also Figure 4, below). It is interesting to note that early studies found that injection of CHX into rats led to activation of S6K1 in the liver [42].

To test whether other inhibitors of protein synthesis exerted similar effects, we also treated cells with emetine or anisomycin. At the concentrations used, anisomycin (10 μ g/ml) and emetine (50 μ g/ml) inhibited protein synthesis by 96 \pm 1% and 92 \pm 2% respectively. Similar to the situation observed for CHX, these inhibitors had little effect on the basal mobility of S6K1 or its phosphorylation at Thr³⁸⁹, but did allow insulin to induce marked increases in phosphorylation as seen with the anti-(phospho-Thr³⁸⁹S6K1) antibody or as judged by the electrophoretic mobility of S6K1 (Figure 1A). Treatment of amino-acid-deprived cells with CHX, emetine or anisomycin also allowed insulin to increase the phosphorylation of rpS6 (Figure 1A), showing that insulin does indeed increase S6K activity within the cells under these conditions. A fourth protein synthesis inhibitor, puromycin, was also tested and found to exert similar effects to those of the three compounds discussed above (results not shown). Thus four different protein synthesis inhibitors have similar effects on the responsiveness of S6K1 and rpS6 phosphorylation to insulin in CHO cells.

We also studied the effects of protein synthesis inhibitors on the regulation of another target for mTOR signalling, 4E-BP1. We examined its binding to eIF4E and also the association of eIF4E with eIF4G, since the binding of 4E-BP1 or eIF4G to eIF4E is mutually competitive [43]. This can be studied by affinity chromatography using m⁷GTP-Sepharose, which retains eIF4E and associated proteins. These can then be analysed by SDS/PAGE and Western blotting. As reported previously [20,21,39], substantial binding of eIF4G to eIF4E can already be seen

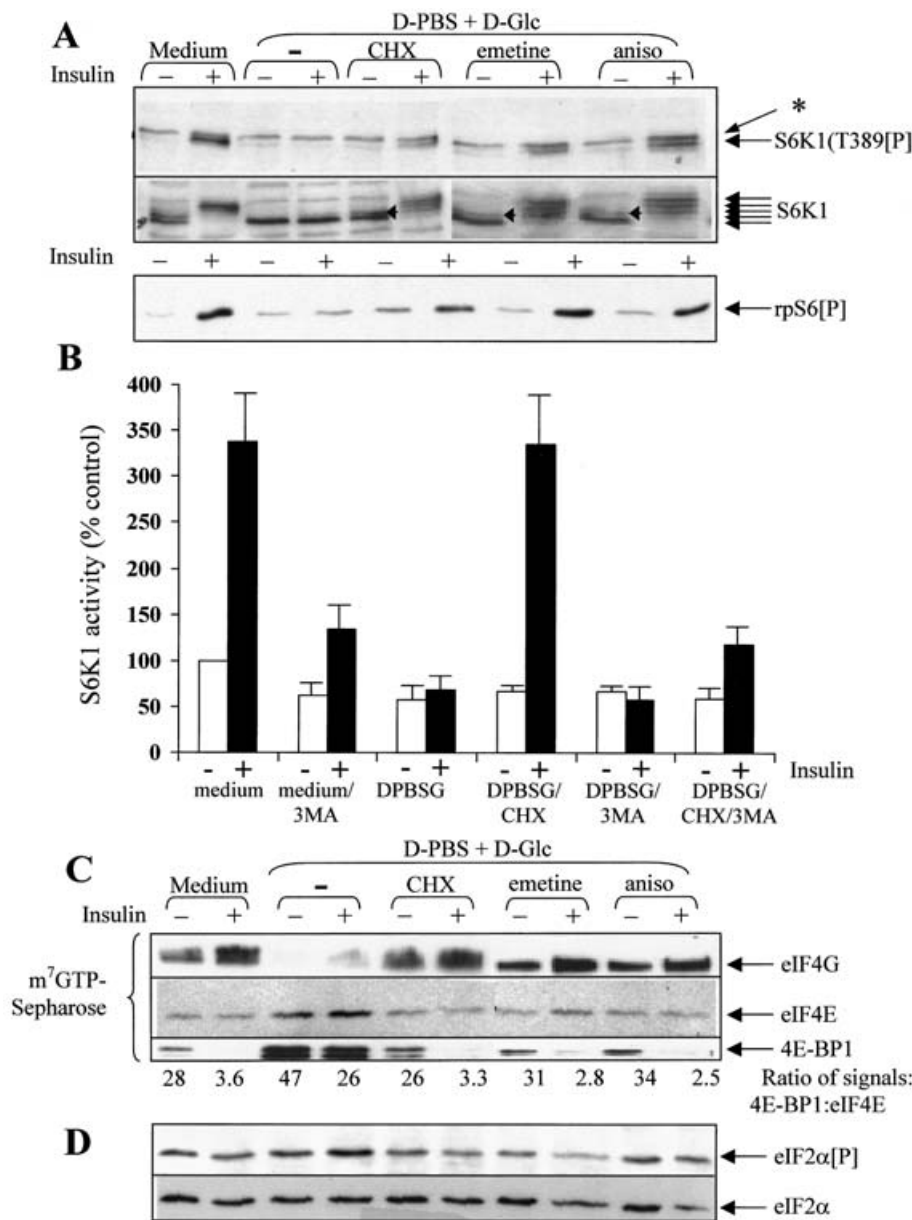


Figure 1 Effects of protein synthesis inhibitors on the phosphorylation of S6K1 and the function of 4E-BP1

CHO.K1 cells were either kept in serum-free Ham's F12 medium (Medium) or transferred to D-PBS/glucose (D-PBS+D-Glc) for 1 h, as described in the Materials and methods section. The indicated protein synthesis inhibitors were added for 30 min prior to the incubation of the cells in the absence (–) or presence (+) of insulin for the final 10 min before being lysed and cytoplasmic extracts prepared. **(A)** Samples were subjected to SDS/PAGE and immunoblotting, and blots were developed with an anti-S6K1 antibody, an antibody that recognizes S6K1 when phosphorylated at Thr³⁸⁹ {S6K1(T389[P])}, or an antibody that reacts with rpS6 when phosphorylated at Ser²³⁵ (rpS6[P]). The asterisk in the top panel denotes a cross-reacting band that is not S6K1, which it is not seen in S6K1 immunoprecipitates (results not shown). The series of arrows in the middle panel indicate that multiple phosphorylated species of S6K1 are resolved: slower migration corresponds to higher states of phosphorylation and activity. **(B)** CHO.K1 cells were treated as indicated and samples containing equal amounts of cellular protein were subjected to immunoprecipitation with anti-S6K1 antiserum. Kinase activity was then assayed using a standard 32-amino-acid peptide, as described in the Materials and methods section. Results are expressed as percentage ± S.D. of control cells in Ham's F12 medium without insulin (*n* = 3). In some cases, cells were treated with 3-MA (1 h, 10 mM final concentration) prior to the addition of insulin. Other additions were as indicated. DPBSG, cells transferred to D-PBS/glucose. **(C)** CHO.K1 cells were treated as indicated and samples of lysates of cells were subjected to affinity chromatography on m⁷GTP-Sepharose and bound material was subjected to SDS/PAGE and immunoblotting using antisera to eIF4G, eIF4E (serves as loading control) or 4E-BP1 as indicated. The signals for 4E-BP1 and eIF4E were quantified densitometrically and are presented as a ratio (this does not give the actual ratio of 4E-BP1 binding to eIF4E). **(D)** CHO.K1 cells were treated as indicated and then equal amounts by protein were analysed by SDS/PAGE and Western blotting using antisera to eIF2α phosphorylated at Ser⁵¹ (eIF2α[P]) or, as loading control, an antibody recognizing both phosphorylated and non-phosphorylated eIF2α. antibody. aniso, anisomycin.

in CHO cells kept in Ham's F12 medium even without insulin treatment (Figure 1C). Insulin led to the dissociation of the small amount of the 4E-BP1 bound to eIF4E and a concomitant modest increase in the binding of eIF4G to eIF4E.

The 4E-BP1 associated with eIF4E in cells kept in Ham's F12 medium is in the partially phosphorylated β-form (Figure 1C). Earlier work [20,21] has shown that the hyperphosphorylated form of 4E-BP1 does not bind to eIF4E in CHO cells, whereas the

less phosphorylated α - and β -forms can. In amino-acid-deprived cells, more 4E-BP1 is bound to eIF4E, and much of this is in the least phosphorylated α -form (which is almost undetectable in m⁷GTP-pull-downs from cells kept in Ham's F12 medium). As reported previously [21,39], insulin failed to induce the complete release of 4E-BP1 from eIF4E in CHO cells kept in D-PBS/glucose. Addition of any of the protein synthesis inhibitors tested to amino-acid-deprived cells led to a modest decrease in the amount of 4E-BP1 associated with eIF4E (Figure 1C). This probably reflects the permissive effect of glucose on this response, which we have noted previously [21]. In the presence of the translation inhibitors, insulin was able to induce the release of almost all the residual bound 4E-BP1. This effect was mirrored in the behaviour of eIF4G. Upon addition of the protein synthesis inhibitors, its association with eIF4E increased to levels seen in amino-acid-replete cells, and was increased further in response to insulin, as seen for cells kept in Ham's F12 medium (Figure 1C). Similar data were obtained for puromycin, in the presence of which insulin again increased the phosphorylation of 4E-BP1 in amino-acid-starved cells (results not shown).

These findings show that each of the four protein synthesis inhibitors tested were able to increase the level of eIF4F complexes and to facilitate the phosphorylation and activation of S6K1 by insulin. Importantly, these inhibitors block protein synthesis through different mechanisms rendering it unlikely that the effects observed reflect either the way in which protein synthesis is perturbed or a non-specific effect of the inhibitors. These compounds inhibit the consumption of amino acids within the cells. One possible mechanism by which these compounds could exert this permissive effect on the regulation of targets of mTOR signalling is therefore by increasing the intracellular concentrations of amino acids and this idea was explored further in subsequent experiments.

The phosphorylation of the α -subunit of eIF2 plays an important role in controlling translation initiation [44] and can be regulated by amino acid availability, e.g. in yeast, where the eIF2 α kinase GCN2 (general control of amino acid biosynthesis, non-derepressing, 2) can be activated by uncharged tRNA. GCN2 homologues have now been found in mammalian cells [45,46]. To study whether the manipulations used in the present study affected the phosphorylation of eIF2 α , we made use of a phospho-specific antibody that recognizes eIF2 α only when phosphorylated at Ser⁵¹ [29], the regulatory site [47]. As a loading control, we performed immunoblots with an antibody that binds eIF2 α whether or not it is phosphorylated. A small increase in eIF2 α phosphorylation was observed upon withdrawal of amino acids (Figure 1D), suggesting that mammalian GCN2 may be activated over the periods of amino acid withdrawal used in the present study. Conversely, treatment of cells with CHX or emetine, which should, if anything, decrease levels of uncharged tRNA and decrease GCN2 activity, caused a small decrease in eIF2 α phosphorylation. However, the magnitude of the effects was small compared with the very marked changes in eIF2 α phosphorylation that are observed in CHO cells under other conditions (for example, see [39]), making it hard to draw a firm conclusion from these data. They nevertheless suggest that the above treatments may alter intracellular amino acid levels.

Effects of protein synthesis inhibitors appear not to be mediated via stress-activated kinase cascades

A well-known effect of anisomycin, at least in some cell types (for example, see [48,49]), is to stimulate stress-activated signalling pathways. Therefore it was also possible that emetine and CHX

stimulate such pathways in CHO cells, and that this effect, through unknown mechanisms, leads to the activation of S6K1 and phosphorylation of 4E-BP1 in amino-acid-deprived cells. There are two well-characterized types of stress-activated protein kinase cascades that might play a role here, the p38 MAP kinase and JNK cascades [50]. None of the four protein synthesis inhibitors tested in the present study detectably activated JNK, as assessed by a direct assay using GST-c-Jun as substrate (Figure 2A). This was surprising in view of the earlier reports [48,49] and we therefore performed an appropriate positive control to verify that the JNK assay detects increases in its activity in CHO-cell extracts. As expected, we observed that arsenite treatment markedly activated JNK (Figure 2A), thereby validating the assay. Arsenite is a powerful activator of JNK and we cannot absolutely rule out the possibility of a very small degree of activation of JNK by protein synthesis inhibitors in CHO cells.

To test the role of p38 MAP kinase α/β , we used SB203580, a specific inhibitor of these enzymes [51]. If these kinases were involved in the effects of protein synthesis inhibitors on S6K1 or 4E-BP1, we would expect this inhibitor to block the effects. SB203580 did not impair the ability of CHX or anisomycin to promote the insulin-induced phosphorylation of either S6K1 (band-shift; Figure 2B) or rpS6 (Figure 2C), or the release of 4E-BP1 from eIF4E (Figure 2D). In fact, if anything, SB203580 tended to enhance these effects, especially in the case of anisomycin (Figures 2C and 2D). To confirm that the compound was indeed effective in blocking p38 MAP kinase signalling in CHO.K1 cells at the concentration used in the present study, we assayed a downstream kinase MAPKAPK2 using hsp25 as substrate (Figure 2E). As a positive control for the assay, we verified that MAPKAPK2 was activated by treatment of cells with arsenite. Some basal MAPKAPK2 activity was observed and, as expected, this was eliminated by treatment of cells with SB203580, showing this assay is a specific readout of activity through the p38 MAP kinase α/β pathway. However, MAPKAPK2 activity was not increased by either incubating cells in D-PBS/glucose, as reported previously [20], or by anisomycin (Figure 2E), and it actually decreased in response to CHX (results not shown). Taken together, these data strongly suggest that neither the effects of amino acid withdrawal nor those of anisomycin or CHX are mediated through activation of the p38 MAP kinase pathway. The effects of the protein synthesis inhibitors on targets of mTOR signalling are therefore unlikely to be due to activation of either of the stress-stimulated kinase cascades examined in the present study.

Translation inhibitors affect S6K1 and 4E-BP1 at concentrations at which they block protein synthesis

In the above experiments, we used concentrations of the protein synthesis inhibitors that were chosen on the basis of their ability to almost completely inhibit protein synthesis. In order to establish whether their effects on protein synthesis correlated with their ability to facilitate mTOR signalling, we performed dose-response experiments. We examined a range of concentrations of each inhibitor for their effects on translation and selected concentrations that gave appreciable, but incomplete, inhibition for further study. Figure 3 shows data for anisomycin and CHX, although similar findings were also obtained for emetine (results not shown). The concentrations chosen were 0.03 and 0.19 $\mu\text{g/ml}$, which gave $30 \pm 10\%$ and $62 \pm 2\%$ inhibition of protein synthesis for CHX and $23 \pm 12\%$ and $63 \pm 8\%$ inhibition for anisomycin respectively. It can be seen from Figure 3(A) that, at these concentrations, CHX and

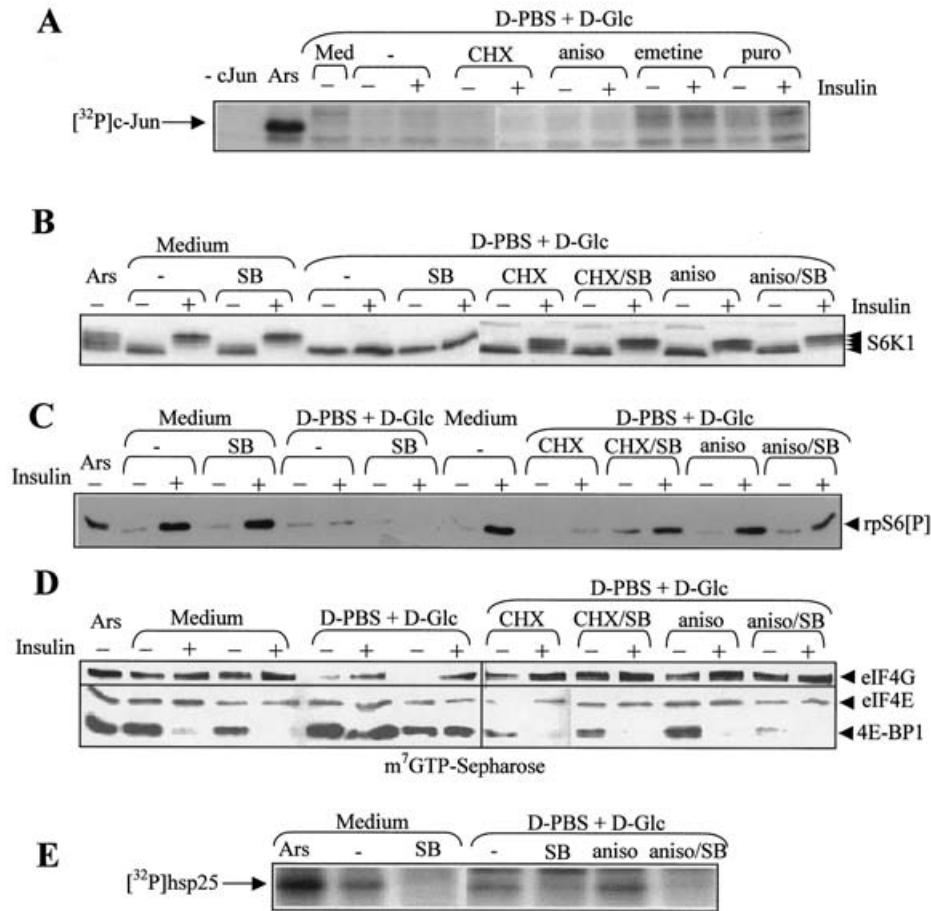


Figure 2 Effects of protein synthesis inhibitors are not due to a stress response

CHO.K1 cells were treated as described in the legend for Figure 1 and the Material and methods section. (A) Samples of extracts from cells treated as indicated were assayed for JNK activity against GST-c-Jun[1–135] using [γ - 32 P]ATP. A representative autoradiograph of the fixed gel is shown. The position of radiolabelled GST-c-Jun is indicated. Ars, arsenite, positive control for stress kinase activation; puro, puromycin; and aniso, anisomycin. -c-Jun, assay performed without GST-c-Jun (negative control). (B–D) Cell treatments were as described with cells being treated with SB203580 (SB), CHX or anisomycin (aniso). SDS/PAGE and Western-blot analysis of S6K1 (B) and phosphorylated rpS6 (C) were performed on equal amounts (by protein) of cell lysates or, for the association of eIF4G and 4E-BP1 with eIF4E, after m^7 GTP-Sepharose chromatography (D). In (B), arrowheads indicate multiple differentially phosphorylated species. In the lower panel of (D), the membrane was probed simultaneously for eIF4E and 4E-BP1. (E) Samples of extracts from cells treated as indicated were assayed for MAPKAPK-2 activity using hsp25. The position of labelled hsp25 is indicated. A representative autoradiograph of the fixed gel is shown.

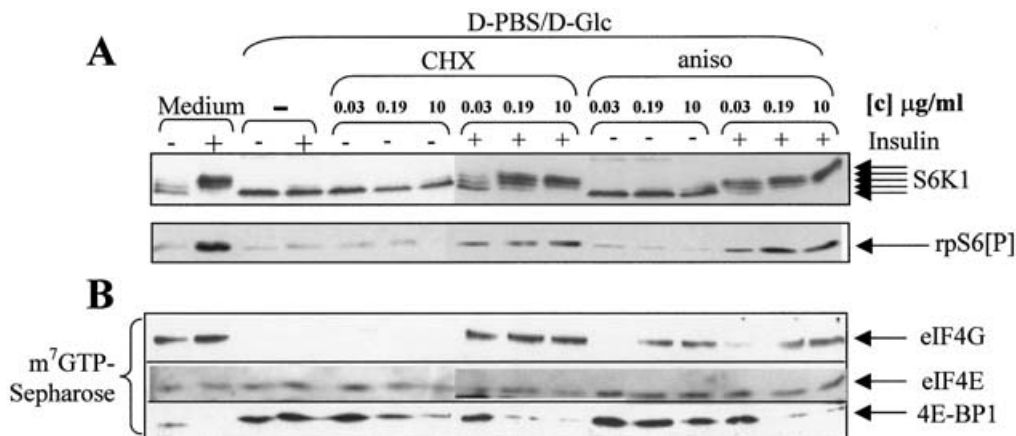


Figure 3 Dose-response data for the effects of protein synthesis inhibitors on S6K1 and 4E-BP1

CHO.K1 cells were treated as described in the legend for Figure 1, but the indicated concentrations of CHX or anisomycin (aniso) were used. (A) Western blots using anti-(S6K1) and anti-(phospho-rpS6) rpS6[p] antibodies. (B) Western blots using antisera to eIF4G, eIF4E or 4E-BP1 as indicated was used to determine the material bound to m^7 GTP-Sepharose. The positions of eIF4G, eIF4E (loading control) and 4E-BP1 are indicated.

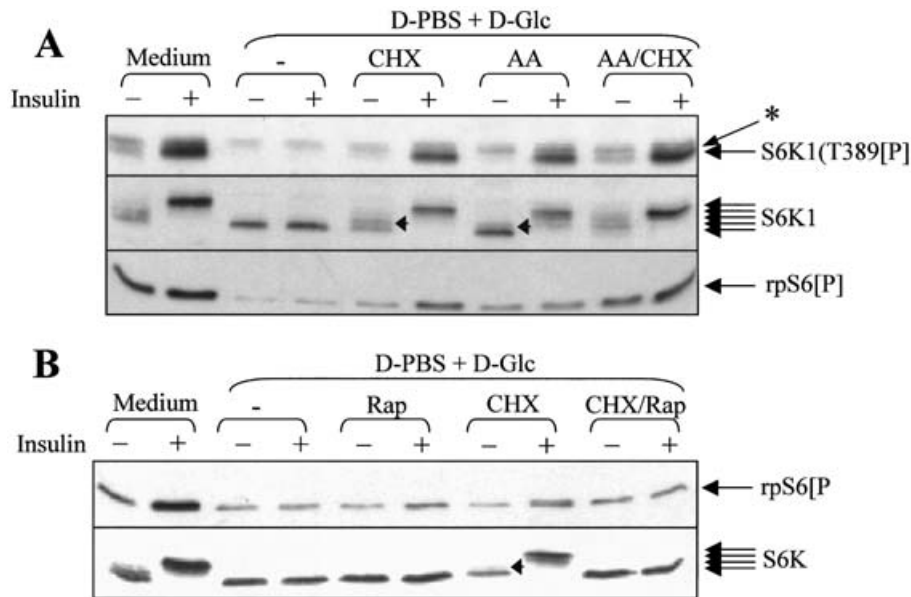


Figure 4 Amino acids and CHX have similar effects on S6K1 and 4E-BP1

CHO.K1 cells were treated as described in the legend to Figure 1, with the following modifications. Cells were pretreated for 30 min with CHX and/or amino acids (AA) (A) or with CHX and/or rapamycin (Rap; 100 nM) (B), prior to treatment in the absence (–) or presence (+) of insulin for 10 min. The cells were lysed, cellular proteins were extracted and analysed by SDS/PAGE and Western blotting using the antisera as described in the legend to Figure 1. The arrowhead indicates the minor more slowly migrating form of S6K1 that appears upon CHX treatment.

anisomycin only facilitated a partial effect of insulin in increasing the phosphorylation of S6K1 or rpS6 (compared with the effect of 10 μ g/ml CHX used earlier). Similarly, at the lowest concentration, CHX failed to allow full release of 4E-BP1 from eIF4E, although increased binding of eIF4G to eIF4E was observed, presumably because some of the 4E-BP1 is released under this condition (Figure 3B). Interestingly, at the middle and highest concentrations used, anisomycin was able to induce some binding of eIF4G to eIF4E, and probably, although this is less easy to see because of the potency of the anti-(4E-BP1) antibody, a partial release of 4E-BP1, even without insulin treatment. This is reminiscent of the behaviour of 4E-BP1 in response to providing CHO cells with amino acids in that amino acids promote the almost complete release of 4E-BP1 and thus also eIF4F assembly, even in the absence of insulin, whereas insulin is required for the full activation of S6K1 ([39] and Figure 1B). Thus CHX and anisomycin only affect targets of mTOR signalling at concentrations that substantially inhibit protein synthesis and, on this basis, their effects on mTOR signalling seem to be distinct from some effects of anisomycin, e.g. on stress-activated kinases, which can occur at very low concentrations [48].

Effects of CHX on S6K1 resemble those of resupplying amino acids and are blocked by rapamycin

The data described above are of particular interest in view of earlier reports that led to the conclusion that amino acid availability was an important positive regulator of S6K1 and 4E-BP1/eIF4F (reviewed in [1,19]). For example, the effects of the protein synthesis inhibitors are qualitatively similar to our previous data [39] for the effects of amino acid addition on the binding of eIF4E to 4E-BP1 or eIF4G in CHO.T cells, which overexpress the human insulin receptor. We considered it important to verify that the effects of amino acids are similar

in the CHO.K1 cells used in the present study. As shown in Figure 4(A), adding amino acids or treatment with CHX had only a small effect on phosphorylation of S6K1, as assessed by its overall electrophoretic mobility, although a faint band above the fastest migrating species became apparent in both cases (indicated by the arrowhead in Figure 4A). Addition of CHX together with amino acids enhanced the basal levels of phosphorylation of both S6K1 and rpS6 more markedly, and may also promote more effectively the effect of insulin as compared with either treatments alone (Figure 4A). Since Thr³⁸⁹ is known to be an important rapamycin-sensitive phosphorylation site in S6K1 (see [2]), it seemed likely that both effects (CHX and amino acids) were mediated through mTOR. Consistent with this, the ability of CHX to promote the insulin-induced phosphorylation of S6K1 (mobility shift) was blocked by rapamycin (Figure 4B), indicating an essential requirement for mTOR signalling in the ability of CHX to promote phosphorylation of S6K1. Similarly, rapamycin blocked the ability of CHX to promote the phosphorylation of 4E-BP1 and its release from eIF4E (results not shown).

Protein synthesis inhibition increases intracellular amino acid levels

Amino acid levels within the cell depend upon the balance between supply (from extracellular sources or protein degradation within the cell) and consumption (e.g. by protein synthesis). It appeared possible that, whereas depriving cells of external amino acids might significantly decrease intracellular amino acid levels, inhibition of protein synthesis might increase their concentrations, since their consumption by translation would be blocked, while their production from protein breakdown would continue. BCAA, especially leucine, are the main regulators of the mTOR pathway in most cells [1,19] and, indeed, leucine is the only amino acid that, when given alone, affects the phosphorylation of 4E-BP1 in CHO.T cells [20]. We therefore assessed the intracellular levels

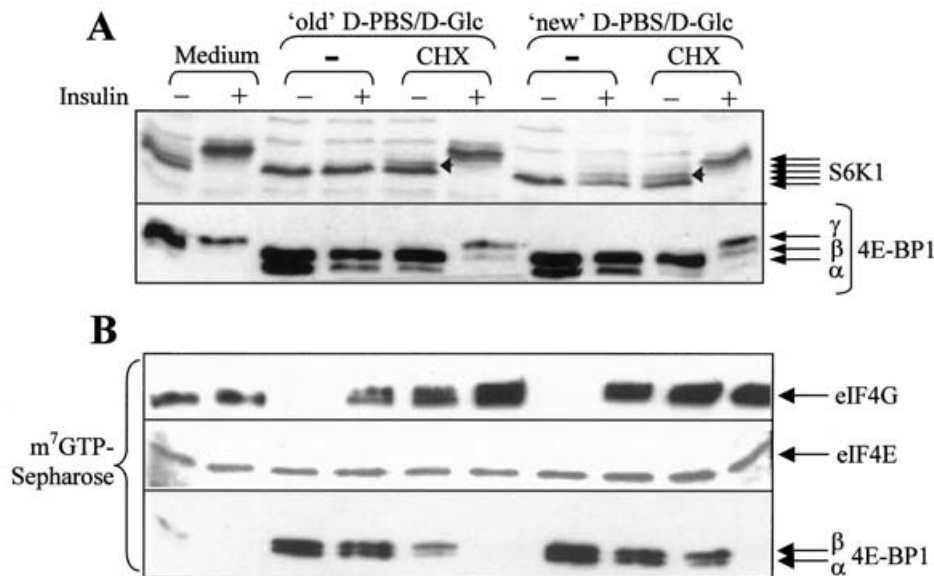


Figure 5 Changing the medium immediately before adding insulin does not reduce the effect of CHX treatment

CHO.K1 cells were treated as described in Figure 1 with the protein synthesis inhibitors in the absence (–) or presence (+) of insulin before being lysed and cytoplasmic extracts prepared. 'old' D-PBS/D-Glc indicates that cells were kept in the same D-PBS/glucose medium up to and including the period of insulin treatment. 'new' D-PBS/D-Glc indicates that this medium was exchanged for fresh D-PBS/glucose immediately before insulin was added for the final 10 min. **(A)** Samples of cell lysate were analysed directly by SDS/PAGE and Western blotting to resolve the differentially phosphorylated forms of S6K1 (indicated by arrowheads) or 4E-BP1 (indicated by α – γ). The arrowhead indicates the minor, more slowly, migrating form of S6K1 that appears upon CHX treatment. **(B)** Samples of cell lysates were subjected to affinity chromatography on m^7 GTP–Sepharose and the bound material was analysed by SDS/PAGE and Western blotting. Positions of eIF4G, eIF4E (loading control) and 4E-BP1 (α - and β -forms) are indicated. The annotation indicating the conditions used for **(A)** also applies to **(B)**.

of BCAA in CHO.K1 cells. BCAA levels in cells transferred to D-PBS/glucose for 1 h were much lower than in cells kept in medium. Setting the level in such control cells at 100%, the amount of BCAA in cells maintained in DPBS/glucose fell to $22 \pm 12\%$ of that value ($n = 3$, for duplicate determinations in each case). This finding indicates that deprivation of amino acids over the times used in the present study does substantially deplete the intracellular pool of BCAA. Treatment of amino-acid-deprived cells for 30 min with CHX resulted in a marked rise in intracellular BCAA levels, almost to the levels seen in the control cells (to $85 \pm 7\%$ of controls, $n = 3$, duplicate measurements). These findings are consistent with the notion that CHX promotes phosphorylation of targets of mTOR signalling by increasing intracellular amino acid levels.

It nevertheless remained theoretically possible that CHX treatment might also increase the amino acid concentrations in the medium and that it was these external amino acids (rather than intracellular ones) that actually enhanced mTOR signalling in CHX-treated cells. To examine this possibility, we tested the effect of changing the medium immediately before adding insulin to CHX-treated amino-acid-starved CHO cells. As shown in Figure 5(A), this manipulation did not adversely affect the ability of insulin to promote phosphorylation of S6K1 or 4E-BP1. It also did not affect the basal level of eIF4G/eIF4E binding or the ability of insulin to promote this further in amino-acid-starved cells treated with CHX (Figure 5B). We also found that CHX did not cause any significant increase in the very low level of amino acids present in the D-PBS/glucose in which these cells are maintained.

Effects of inhibiting protein degradation

The most likely explanation for the effects of the protein synthesis inhibitors on S6K1 and 4E-BP1 is therefore that they shift the balance of protein synthesis and degradation within the cell to

favour the accumulation of intracellular amino acids and thus enhance mTOR signalling. If, as seems likely, the increase in amino acid pools that occurs when protein synthesis is inhibited is due to continuing protein turnover, then blocking protein degradation should counter the effects of, for example, CHX.

To test this, we used 3-MA [52], a widely used inhibitor of autophagy, which is a major form of protein breakdown. Amino-acid-fed or -deprived cells were incubated in the absence or presence of 3-MA and with or without CHX prior to exposure to insulin. Treatment of amino-acid-fed or -starved cells with 3-MA caused a decrease in the phosphorylation of 4E-BP1 as shown by the increased proportion of the eIF4E-bound 4E-BP1, which was in the least phosphorylated α -form relative to the situation in the corresponding controls (Figure 6A, compare lanes 1 and 3, and lanes 5 and 9). CHX increased the phosphorylation state of 4E-BP1, as shown by the higher proportion of the protein in the β -form in CHX-treated cells as compared with the appropriate controls (Figure 6A, compare lanes 5 and 7). 3-MA attenuated the ability of CHX to promote the phosphorylation of 4E-BP1 as indicated by the fact that the protein was mainly in its β -form in CHX-treated cells, but mostly as the α -isoform in cells treated with CHX and 3-MA (Figure 6A, compare lanes 7 and 11). 3-MA also partially prevented the insulin-induced release of 4E-BP1 from eIF4E normally observed in amino-acid-deprived cells treated with CHX. 3-MA largely reversed the ability of CHX to allow insulin to increase the overall phosphorylation of S6K1, as judged from its mobility (Figure 6A) and the phosphorylation of Thr³⁸⁹ (Figure 6B). Treatment of CHO.K1 cells with anisomycin or emetine allowed insulin to increase the phosphorylation of 4E-BP1, which migrated entirely as the most phosphorylated γ -form under this condition (Figure 6C). Treatment with 3-MA impaired this effect, with a significant proportion of the 4E-BP1 running as the least phosphorylated β -form even after insulin stimulation of the cells. 3-MA also repressed the basal activity of S6K1 in cells

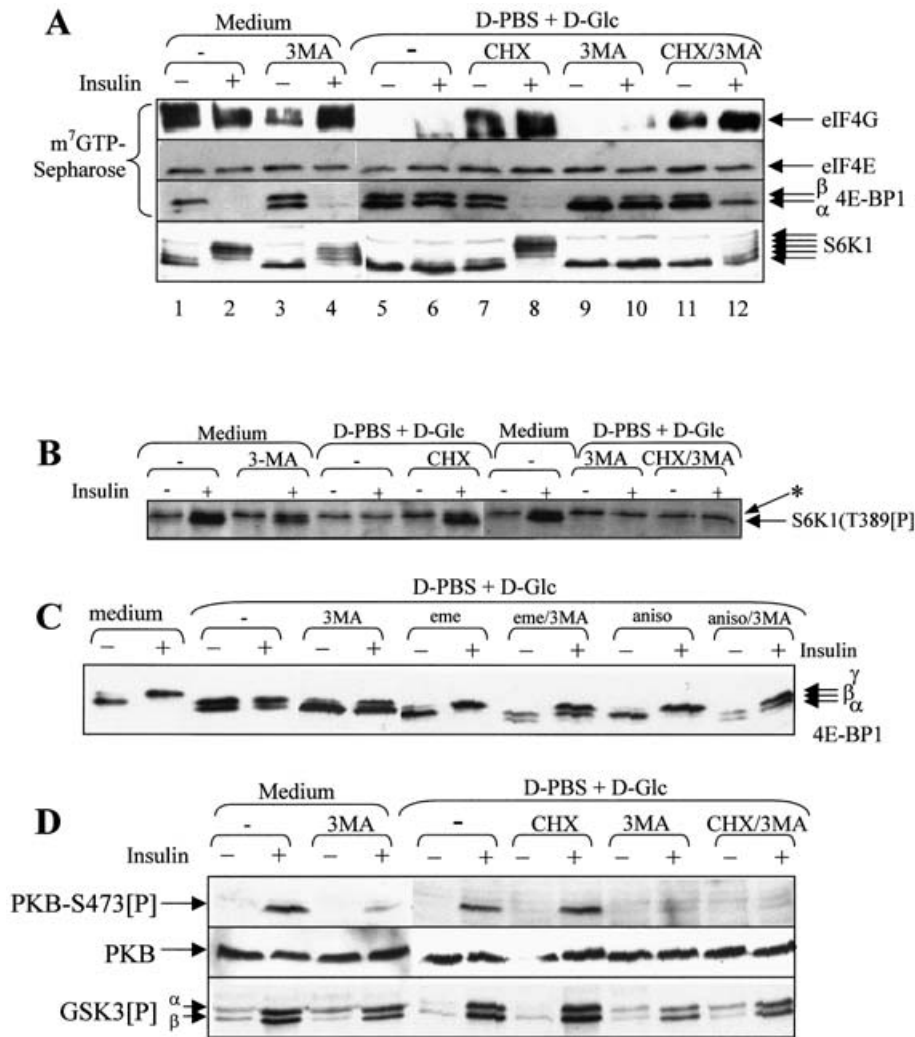


Figure 6 3-MA attenuates the effect of CHX

CHO.K1 cells were treated as described in Figure 1 with the protein synthesis inhibitors [CHX, emetine (eme), anisomycin (aniso) and/or 3-MA (2 h, 10 mM)] in the absence (–) or presence (+) of insulin before being lysed and cytoplasmic extracts prepared. **(A)** Extracts were either subject to affinity chromatography on m^7 GTP-Sepharose (as indicated) prior to gel electrophoresis and immunoblotting, or analysed in this way directly (bottom section). The positions of the proteins studied are shown (α - and β -forms of 4E-BP1 are indicated). **(B)** Cell lysates were directly subjected to SDS/PAGE and immunoblotting with the anti-phospho-S6K1 (Thr³⁸⁹) antibody. The asterisk denotes a non-specific band. **(C)** Analysis was by SDS/PAGE and Western blotting for 4E-BP1 using gels containing 13.5% (w/v) acrylamide and 0.36% (w/v) methylene bisacrylamide. The positions of the α -, β - and γ -forms of 4E-BP1 are indicated. **(D)** Samples of extracts of cells treated as indicated were analysed by SDS/PAGE and Western blotting using antisera that recognise PKB when phosphorylated at Ser⁴⁷³ (PKB[P]), PKB irrespective of its state of phosphorylation (PKB, as loading control) or phosphorylated GSK3 (GSK3[P]). Two bands were observed for phosphorylated GSK3, as the antibody used recognizes both the α - and β -isoforms present in CHO cells.

maintained in Ham's F12 medium, although insulin was still able to elicit substantial activation of S6K1 in cells kept in this medium containing 3-MA (Figure 6B). This last finding could suggest that protein breakdown contributes significantly to the intracellular amino acid pool even in cells in normal growth medium (but see also below). As reported above, insulin did not activate S6K1 in amino-acid-deprived cells and was also unable to do so in the presence of 3-MA. When such cells are also treated with CHX, it was clear that 3-MA substantially prevented the activation and phosphorylation (mobility shift) of S6K1 normally seen in amino-acid-deprived cells given CHX (compare Figure 6A and Figure 2B).

We were concerned that the inhibition by 3-MA of the activation of S6K1 might reflect possible effects on this compound on other signalling pathways that impinge on S6K1. To test whether 3-MA affected other signalling pathways related to the regulation of mTOR signalling, we examined whether it affected the ability

of insulin to activate PKB, which has been implicated by several studies in the upstream control of 4E-BP1 and, in some cases, S6K1 in response to insulin [53–57]. 3-MA substantially impaired the ability of insulin to activate PKB, as demonstrated using phospho-specific antibodies that recognize PKB only when it is phosphorylated at Ser⁴⁷³ (Figure 6D) or Thr³⁰⁸ (results not shown), sites known to be critical for its activation. This ability of 3-MA to impair activation of PKB might explain, in part, why it interferes with the activation of S6K1 and 4E-BP1. However, it cannot explain the inhibition of basal 4E-BP1 and S6K1 phosphorylation, since PKB is not detectably active under this condition (Figure 6D, and [40]). It should also be noted that the effect of 3-MA on the phosphorylation of GSK3 (Figure 6D) is much smaller than its effect on PKB, presumably as a result of signal amplification downstream of PKB, indicating that the effect of 3-MA on insulin-induced phosphorylation of 4E-BP1 and S6K1 may only be due, in part, to this effect on PKB signalling.

DISCUSSION

The results in the present study for CHO.K1 cells confirm previous findings that, for CHO.T cells, the presence of amino acids in the medium promotes the phosphorylation of S6K1 and the release of 4E-BP1 from eIF4E [20,39]. S6K1 is relatively dephosphorylated and inactive in CHO.K1 cells deprived of external amino acids and adding amino acids both increases its basal phosphorylation and permits its activation by insulin. In the case of 4E-BP1, adding amino acids promotes its phosphorylation and concomitant release from eIF4E, allowing the formation of eIF4F complexes containing eIF4E and eIF4G. An important question raised by our previous studies [20] concerns the mechanism by which amino acids exert these effects. In particular, it was not clear whether externally added amino acids exert their effect from outside the cell or have to enter the cell first to do this in order to replenish diminished intracellular pools. A range of studies, including work in CHO cells [20], has demonstrated that BCAA, especially leucine, are particularly effective in stimulating phosphorylation of S6K1 and 4E-BP1 (reviewed in [1,19]). In the present study, we show that intracellular levels of BCAA are indeed markedly decreased in amino-acid-deprived CHO cells.

We show further that inhibiting protein synthesis using any one of four different compounds has a similar effect on S6K1 and 4E-BP1/eIF4F as the addition of amino acids to the medium. The fact that similar effects are seen with four different inhibitors, whose only common feature is their ability to inhibit protein synthesis, strongly suggests that it is their ability to inhibit translation that underlies their effects on 4E-BP1 and S6K1 rather than a 'side-effect'. Importantly, these compounds exert strong effects on S6K1 and 4E-BP1 at concentrations that inhibit protein synthesis, but not at lower ones. This effect is therefore distinct from the ability of anisomycin to stimulate stress-activated protein kinases at concentrations lower than those at which it affects protein synthesis [48]. We also provide further evidence that their effects are not exerted via a stress response involving either the p38 MAP kinase α/β or JNK pathways.

Furthermore, CHX raises the intracellular concentrations of BCAA towards levels seen in amino-acid-replete cells without a detectable increase in the levels of BCAA in the medium. Moreover, when the amino-acid-free medium of the CHX-treated cells was changed for fresh ('naïve') medium immediately before addition of insulin, the hormone was still able to elicit phosphorylation of S6K1 and release of 4E-BP1 to a similar extent as in cells kept for 30 min in D-PBS/glucose and CHX. These findings strongly suggest that the effects of CHX are not due to an accumulation in the D-PBS/glucose of amino acids that have been released from the cells following inhibition of protein synthesis. Such export is, in any case, unlikely in the absence of external amino acids, since the leucine transporter, system L, is an antiporter. It seems more likely that the effects of CHX and the other protein synthesis inhibitors tested on S6K1 and 4E-BP1 are due to accumulation of intracellular amino acids as a consequence of the inhibition of protein synthesis, whereas protein breakdown continues to contribute to the intracellular amino acid pool.

Treatment of amino-acid-deprived cells with the autophagy inhibitor 3-MA attenuated the ability of CHX to promote phosphorylation of S6K1 and 4E-BP1. This is again consistent with the idea that the effect of CHX is to increase intracellular amino acid levels as they continue to be generated by protein breakdown. However, interpretation of these data is made more complex by the observation that 3-MA impairs signalling from the insulin receptor to PKB. Nonetheless, several features suggest that the effect of 3-MA is at least, in part, due to inhibition of protein breakdown. Firstly, 3-MA decreased the basal level of phos-

phorylation of 4E-BP1 in amino-acid-deprived cells, where PKB activity is very low [39]. Second, recent findings [56–58] indicate that activation of S6K1 is not driven by PKB. Third, although 3-MA substantially decreased the phosphorylation of PKB, it had much less effect on a direct substrate for PKB, GSK3, suggesting that there may be substantial amplification within the pathway downstream of PKB that compensates for interference with activation of PKB.

A previous report [59] has suggested that the inhibition of S6K1 seen in amino-acid-deprived cells may be a consequence of accumulation of uncharged tRNA. Evidence for this included the observation that amino acid alcohols, which are competitive inhibitors of aminoacyl-tRNA synthetases, led to inhibition of S6K1. We have studied extensively whether such compounds, specifically histidinol and leucinol, affect mTOR signalling in CHO cells, but we consistently saw no effect (A. Beugnet, X. Wang, J. Patel and C. G. Proud, unpublished work). Other workers [60] have found that amino acid alcohols did not affect 4E-BP1 or S6K1 in adipocytes. This may be because they are rather poor inhibitors of aminoacyl-tRNA synthetases. Dennis et al. [22] found that amino acid starvation did not lead to accumulation of uncharged tRNA in HEK-293 cells. Jousse and Ron (reported in [3]) have found that GCN2 is not required for the regulation of the mTOR pathway by amino acids. Taken together, these data are not consistent with the idea that uncharged tRNA is important in regulating mTOR signalling in these types of cells [59], although it is still conceivable that it does in other cell types. An alternative possibility, suggested by our data (present study) and that of Dennis et al. [22] for mammalian cells, and by the findings of Christie et al. [25] for *Xenopus* oocytes, is that it is the actual intracellular concentrations of certain amino acids (e.g. BCAA) that are the important regulator of the mTOR pathway. Operation of such a mechanism implies the existence of an intracellular amino acid sensor that acts upstream of mTOR. Such an entity still awaits identification and further work is required to achieve this and explore whether intracellular amino acids themselves, or a metabolite derived from them, are the key signalling molecule in mammalian cells. It is possible that amino acids may interact with and modulate the newly discovered protein Raptor [61,62], which interacts with mTOR and plays a key role in signalling from mTOR to S6K1 and 4E-BP1. Recent findings suggest that the proteins hamartin and tuberlin (also termed TSC1/2) regulate mTOR function and there is evidence that they may modulate the control of (m)TOR signalling by amino acids [57,63]. It is also conceivable that TSC1/2 are regulated by intracellular amino acid availability or may themselves sense amino acids. Further work is required to assess these possibilities.

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