Background
The initiation of translation of mRNAs is an important control point in protein synthesis in eukaryotes and requires a set of initiation factors (eIFs). The cap-binding protein eIF4E recognises the 5'cap-structure of the mRNA, and is a component of the eIF4F complex consisting of eIF4E, eIF4G, a scaffolding protein [1,2], and eIF4A, an RNA helicase [3,4]. Any secondary structure in the 5'untranslated region of the mRNA is thought to be unwound by eIF4A together with eIF4B or eIF4H [5]. The 40S subunit of the ribosome binds to the eIF4F complex through an association between eIF4G and eIF3, which interacts
directly with the 40S ribosomal subunit. The preinitiation complex, containing the 40S ribosomal subunit, eIF4F, eIF4B, and Met-tRNA\textsubscript{i}·eIF2\textsubscript{a}·GTP, scans the 5′UTR until the AUG start codon is located. The subsequent hydrolysis of the GTP bound to eIF2 is promoted by eIF5, after which eIF2·GDP leaves the ribosome. The 60S ribosomal subunit can then join and the 80S complex is formed. eIF2 in the GDP-bound state is inactive and, in order to return to the active form again, the GDP is exchanged for GTP in a step promoted by the guanine nucleotide exchange factor eIF2B.

The next stage in the translation process, the elongation step, can be regulated via changes in the activity of eEF2 [6]. Phosphorylation of eEF2 at Thr56 results in its complete inactivation [7].

Human primary T-cells are metabolically quiescent, with little ongoing DNA, RNA or protein synthesis [8–10]. The low protein synthesis rate in quiescent T cells is associated with low levels of initiation factors in these cells. The rate of protein synthesis increase 2–4 fold after 4 h of mitogenic stimulation [11], and it has been reported that the mRNA and protein levels for several translation initiation factors increased during T cell activation. The mRNA levels of eIF4A, eIF2\textsubscript{a}, and eIF4E increased rapidly after stimulation [12]. However, the increase in the levels of the corresponding protein lagged significantly behind. It is therefore likely that increased levels of translation factors contribute to the pronounced stimulation of protein synthesis that occurs during T cell activation at later times, while modulation of the activity of several translation initiation factors e.g. by phosphorylation or association with binding proteins is important in the early phase of T cell activation [13].

Increased phosphorylation of eIF4E in T lymphocytes has been reported under several conditions. Activation of quiescent mature porcine peripheral blood mononuclear cells with phorbol 12-myristate 13-acetate (PMA) or concanavalin A [14] or stimulation of human primary T cells with PHA [15], PMA, or PMA plus ionomycin [16] caused a rapid increase in the phosphorylation of eIF4E. Similarly, stimulation of the T cell receptor in the human leukaemic T cell line Jurkat with OKT-3, or treatment with PMA, increased eIF4E phosphorylation [14,17], and a significant increase in the amount of eIF4F complexes was also detected.

The activity of eIF4E can also be modulated by its association with eIF4E-binding proteins, of which 4E-BP1 is the best-studied. Phosphorylation of 4E-BP1 leads to its dissociation from eIF4E, leaving eIF4E free to bind eIF4G and form eIF4F complexes [18,19]. In a murine cytotoxic T cell line, interleukin-2 induced the phosphorylation of 4E-BP1 [20]. 4E-BP1 is present in human primary T lymphocytes [21] and becomes phosphorylated in response to PMA or PMA plus ionomycin [16].

In several cell lines, an increase in eIF2B activity coincides with an increase in protein synthesis [22,23]. One mechanism to regulate the activity of eIF2B is via phosphorylation of its ε-subunit (eIF2B\textsubscript{ε}) by GSK-3, which causes a decrease in eIF2B activity [24]. Stimulation of T cells with PMA plus ionomycin caused a rapid rise in eIF2B activity, which coincided with inactivation of GSK-3 [25], suggesting a role for dephosphorylation of eIF2B. The activity of eIF2B can also be modulated by phosphorylation of the α-subunit of eIF2. eIF2 phosphorylated in its α-subunit acts as a competitive inhibitor of eIF2B [26]. Stimulation of T cells with PHA did not cause significant changes in the phosphorylation state of eIF2\textsubscript{α} [15], excluding this mechanism of regulation under this condition.

In this study we used the antibodies, αCD3 and αCD28, to activate resting human primary T lymphocytes. Engagement of αCD3 activates the T cell receptor, while cross-linking of αCD28 with the B7 receptor will supply a co-stimulatory signal, which is required for full activation of a resting T cell [27,28]. We have studied the effects of T cell activation on protein synthesis and on the activities and/or phosphorylation states of several translation initiation factors. Furthermore, the signalling pathways involved in these changes have been investigated.

**Results**  
**Activation of primary T cells with a physiological stimulus increased protein synthesis**

We activated T cells with the antibodies, αCD3 and αCD28, for up to 24 h and measured protein synthesis and the activation of several signalling pathways that are important for the regulation of translation factors and that have been shown to increase after activation of T cells [29] (Fig. 1).

The rate of protein synthesis in resting T cells is low, and activation of the cells with αCD3 and αCD28 led to a substantial increase in the incorporation of [\textsuperscript{35}S]methionine into protein. Within 24 h of activation, the rate of protein synthesis was increased 6-fold (Fig. 1A), depending on the blood donor.

After 30 min of activation, phosphorylation of ERK and p38 MAPK, and the activity of PKB were measured. Phosphorylation of ERK2 and p38 MAPK increased already after 5 min and reached a maximum after 30–60 min of treatment (data not shown). After 30 min of treatment, a clear phosphorylation of ERK and p38MAPK was detected (Fig. 1B). Treatment with PMA, a potent activator of PKC, was used as a positive control. PMA induced, in particular,
Figure 1
Activation of T cells with αCD3 and αCD28. A. Primary T lymphocytes were activated with both αCD3 and αCD28 for 4, 8, 16 or 24 h and for the last 45 min 10 µCi/ml of [35S]-labelled methionine was present. The experiment was performed in duplicate. Incorporation of [35S]-labelled methionine into equal amounts of protein was measured. Protein synthesis in control cells was set at 100%. Methionine incorporation ranged between 1000 and 2000 cpm per 50 µg of protein. (control cells at t = 0 h and t = 24 h are not significantly different, n = 3). B. T cells were activated by αCD3 and αCD28 or with PMA. Cells were activated for 30 min, harvested and 50–80 µg of lysate was analyzed by SDS-PAGE and Western blotting. Antibodies that recognize the phosphorylated form of either ERK (pp42) or p38 MAPK (pp38) were used. Even loading of the gel was verified using anti-ERK2 (p42). Similar results were obtained in three sets of experiments. C. PKB activity was measured as described in Materials and Methods (p<0.05, n=4).
a greater extent of phosphorylation of ERK than \( \alpha \text{CD3} \) plus \( \alpha \text{CD28} \). For p38 MAPK phosphorylation, the difference between these stimuli was less pronounced (Fig. 1B).

An 1.5 fold increase in PKB activity (Fig. 1C) was detected within 30 min of activation of T cells by \( \alpha \text{CD3} \) and \( \alpha \text{CD28} \).

The increase in protein synthesis and the stimulation of various signalling pathways indicated that treatment of T cells with \( \alpha \text{CD3} \) and \( \alpha \text{CD28} \) led to activation of the cells.

**The cellular protein levels of eIF4E and eIF2B\(\text{e}\) do not change during the early phase of T cell activation**

Previous studies using mitogenic stimuli showed that the levels of several initiation factor proteins increase later (>16 h) following T cell activation and probably contribute to the increase in protein synthesis [15,16,25]. To examine whether activation of T cells with \( \alpha \text{CD3} \) and \( \alpha \text{CD28} \) also affected initiation factor levels, the amounts of the cap-binding protein, eIF4E, and of the catalytic subunit of the eIF2B complex, eIF2B\(\text{e}\), were assessed at different time points (Fig. 2). The amounts of eIF4E and eIF2B\(\text{e}\) protein each remained constant during the first 6 h.

In this study, we have focused on the mechanisms underlying the initial response after activation of primary T cells by \( \alpha \text{CD3} \) and \( \alpha \text{CD28} \) and the concomitant increase in protein synthesis. Since the levels of initiation factor proteins did not change in this early phase of T cell activation, we considered the possibility that changes in the phosphorylation state and/or activities of several translation factors were involved in the initial activation of protein synthesis in T cells.

**Protein synthesis is regulated via multiple signalling pathways**

Primary T lymphocytes were activated with \( \alpha \text{CD3} \) and \( \alpha \text{CD28} \), and after 1 h of activation, protein synthesis was increased 1.2 fold (Fig. 3). To study the signalling events involved in this increase in protein synthesis, we performed the experiment in the presence of different specific signal transduction pathway inhibitors (Fig. 3). The increase in overall protein synthesis was consistently blocked by each of the signal transduction inhibitors used, i.e. the PI 3-kinase inhibitor wortmannin, the mTOR inhibitor rapamycin, the p38 MAPK\(\alpha/\beta\) inhibitors SB203580 and SB202190, and the MEK inhibitor PD98059. It appears that the immediate activation of protein synthesis in T cells involves interplay between several signalling pathways.

Stimulation of T cells with the more potent stimulus PMA for 1 h led to a substantially larger increase in protein synthesis (1.8 fold) compared to activation with \( \alpha \text{CD3} \) and \( \alpha \text{CD28} \) (Fig. 3).
Phosphorylation of eIF4E, eIF4F complex formation and 4E-BP1 phosphorylation remain unchanged after T cell activation

Phosphorylation of the cap-binding protein eIF4E can be regulated via the ERK and p38 MAPKα/β pathways [17,30], two pathways that appear to be important for the regulation of protein synthesis in T cells (Fig. 3). Furthermore, the phosphorylation of eIF4E has been reported to be increased in response to several different treatments of primary T lymphocytes [15,16] or the Jurkat T cell line [14,17]. However, activation of T cells with αCD3 and αCD28 for up to 60 min did not cause a significant change in the phosphorylation state of eIF4E (Fig. 4A). Stimulation of T cells with either αCD3 or αCD28 alone was also insufficient to change the phosphorylation state of eIF4E (data not shown). We did detect a marked change in phosphorylation of eIF4E after 30 min of PMA treatment, indicating that the cells respond to this stimulus (Fig. 4A). In addition, treatment of the Jurkat T cell line with αCD3 and αCD28 caused phosphorylation of eIF4E that was already detectable after 30 min, demonstrating the effectiveness of the antibodies (αCD3 and αCD28) used (Fig. 4A).

An important way of regulating eIF4F assembly is through eIF4E-binding proteins such as 4E-BP1. Phosphorylation of 4E-BP1 leads to its release from eIF4E, allowing the latter protein to bind eIF4G [31]. The phosphorylation of 4E-BP1 can be detected by virtue of a reduction in its mobility upon SDS-PAGE [18,19]. As a control to demonstrate that different forms of human 4E-BP1 can be resolved on our gel system, we used HeLa cell extract and in this case three separate bands (α, β and γ) were indeed detected (Fig. 4B), indicative of differently phosphorylated forms. In resting T-cells, 4E-BP1 was mainly present in the unphosphorylated form (α-form) as reported before [32]. We were unable to detect any changes in mobility of 4E-BP1, and therefore its phosphorylation after stimulation of the cells with αCD3 plus αCD28 or PMA (Fig. 4B).

Formation of eIF4F complexes was studied by purification of eIF4E on m7GTP-Sepharose followed by a Western blot to detect associated eIF4G. In resting T cells, eIF4F complexes are already present, and after 1h of activation with αCD3, αCD28, or both antibodies, the amount of eIF4G bound to eIF4E remained unchanged (Fig. 4C). Similar results were obtained after 30 min of activation (data not shown). Surprisingly, no 4E-BP1 associated with eIF4E was detected, even though up to 2 mg of T cell extract was used in a m7GTP Sepharose pull down (data not shown). This could be due to low amounts of 4E-BP1 protein present in resting T cells.

These data indicate that increased formation of eIF4F complexes is not required for the activation of protein synthesis in the early phase of T cell activation.

Regulation of eIF2B activity after activation of T cells

In several cell types, an increase in overall protein synthesis coincides with an increase in eIF2B activity [22,23,25]. We therefore examined the activity of eIF2B after activation of primary T cells (Fig. 5A). After 1 h of activation with αCD3 and αCD28, the activity of eIF2B increased 2.2 fold. The increase in eIF2B activity was not caused by a change in the phosphorylation state of the α-subunit of eIF2 (Fig. 5B) or by an increase in the amount of eIF2B protein (Figs. 2 and 5B and 5E). Immunoprecipitation of different amounts of T cell extracts showed that the eIF2B antibody was able to detect different levels of protein in the immunoprecipitations within the same range used in Fig. 5B (bottom panel). Taken these results together it suggested that eIF2B was regulated directly, e.g. via phosphorylation. To study which signal transduction pathways are involved in the regulation of the activity of eIF2B, the cells were activated in the presence of specific signal transduction pathway inhibitors and eIF2B activity was measured. The basal activity of eIF2B was slightly affected in the presence of the PI 3-kinase inhibitor Wortmannin, the mTOR inhibitor rapamycin, and the p38 MAPKα/β inhibitor SB203580, however the αCD3 plus αCD28-induced increase in eIF2B activity was completely blocked in the presence of each inhibitor. The MEK inhibitor PD98059 did not affect the basal eIF2B activity and was also able to inhibit the αCD3 and αCD28-induced increase in eIF2B activity, showing that all these signalling pathways are required to mediate the activation of eIF2B (Fig. 5A).

Activation of eIF2B after stimulation of the cells with PMA was about 2 fold higher than after stimulation with the antibodies. An increase in eIF2B activity after stimulation of primary T-cells with PMA/ionomycin has been reported before [16,25].

It has been suggested that GSK-3 may be an important regulator of eIF2B activity, i.e. in response to insulin [33,34] and during cell survival [35]. Phosphorylation of eIF2B by GSK-3 inhibits the activity of the eIF2B complex [33]. GSK-3 activity is decreased only by a small extent (15%) after T cell activation with αCD3 and αCD28 (Fig. 5D). In contrast, PMA treatment reduced GSK-3 activity by about 50%, which is similar to previously reported data.

GSK-3 phosphorylates eIF2B on Ser540. Therefore, we analyzed the phosphorylation state of this site using a phospho-specific antibody. We were unable to detect any change in the phosphorylation of this site (Fig. 5E) in response to αCD3 and αCD28, excluding a role for GSK-3.
Figure 4
Regulation of eIF4E phosphorylation and eIF4F formation. A. Jurkat T cells were activated for 30 or 60 min by both αCD3 and αCD28. The primary T lymphocytes were activated for the indicated times with both αCD3 and αCD28 or with PMA. eIF4E was purified using m7GTP Sepharose, analyzed on a one-dimensional iso-electric focusing gel and detected by Western blotting. 4E and 4E-P indicate unphosphorylated and phosphorylated eIF4E respectively. B. 100 µg of total cell lysate from primary T cells treated for 1 h with αCD3 and αCD28 or PMA was analyzed by SDS-PAGE and Western blotting to detect 4E-BP1. (-) indicates untreated cells. The lane with 4E-BP1 from HeLa cell extract was obtained from a shorter exposure from the same blot. C. Formation of eIF4F was analyzed after 60 min activation of primary T cells with either αCD3, αCD28 or both. eIF4E was purified as described above and its association with eIF4G was analyzed by SDS-PAGE and Western blotting. An eIF4E blot was used to verify equal loading of all lanes. Similar results for eIF4E, eIF4G and 4E-BP1 were obtained in three independent experiments.
**Figure 5**

**Regulation of eIF2B activity.**

**A.** T cells were preincubated with wortmannin (W, 100 nM), rapamycin (R, 100 nM), SB203580 (SB, 10 µM), or PD98059 (PD, 50 µM) for 30 min and left untreated (white bar) or the cells were activated with both αCD3 and αCD28 (black bar) for 1 h. Simultaneously cells were activated with PMA (hatched bar) for 1 h. An eIF2B assay was performed as described in Materials and Methods. Bars marked with * are significantly different from the untreated cells (p < 0.05, n = 5).

**B.** Cell lysates from resting and stimulated cells (1 h αCD3 and αCD28) were analyzed by SDS-PAGE and Western blotting to detect phosphorylated eIF2α. eIF2B was immunoprecipitated from 400 µg of lysate using αeIF2Bε and the amount of protein was analyzed by SDS-PAGE and Western blotting. Similar results were obtained in three experiments.

**C.** To test the sensitivity of the eIF2Bε antibody different amounts of T cell extracts (as indicated) were immunoprecipitated with αeIF2Bε and analyzed by SDS-PAGE and Western blotting. Similar results were obtained in two experiments.

**D.** T cells were left untreated (white bar) or the cells were activated with both αCD3 and αCD28 (black bar) or PMA (grey bar) for 1 h. GSK-3α and β were immunoprecipitated together from 400 µg of lysate and a kinase assay was performed as described in Materials and Methods (p<0.05, n=4).

**E.** eIF2Bε was immunoprecipitated from 400 µg of lysate from resting and stimulated cells (1 h αCD3 and αCD28) and the total amount of eIF2Bε and the phosphorylation state of Ser540 were analyzed by SDS-PAGE and Western blotting. Similar results were obtained in two experiments.
in the regulation of eIF2B activity in T cells under these conditions.

**Dephosphorylation of eEF2**

Elongation factor 2 (eEF2) plays an important role in the regulation of the rate of elongation, and therefore in the regulation of the rate of overall protein synthesis. Phosphorylation of eEF2 causes its inactivation [7]. Phosphorylation of eEF2 was rapidly but only transiently decreased after activation of the primary T lymphocytes with αCD3 and αCD28 (Fig. 6). Within 3 min, maximum dephosphorylation was reached and the phosphorylation level returned to a level similar to that of resting T cells by 10 min. Given the transient nature of these changes, it is unlikely that regulation of eEF2 plays a role in the sustained increase in the rate of protein synthesis after activation of T cells. However, dephosphorylation of eEF2 could play a role very early in T cell activation.

**Regulation of p70 S6 kinase upon T cell activation**

Activation of p70 S6 kinase and phosphorylation of the ribosomal protein S6, an in vivo substrate of p70 S6 kinase, coincide with increased translation of specific mRNAs, namely the 5'TOP mRNAs [36]. However, a recent report has questioned the role of p70 S6 kinase in 5'TOP messenger translation [37].

We studied the effect of activation of T cells with αCD3 plus αCD28 on these proteins. The activity of p70 S6 kinase and phosphorylation of the ribosomal protein S6, an in vivo substrate of p70 S6 kinase, coincide with increased translation of specific mRNAs, namely the 5'TOP mRNAs [36]. However, a recent report has questioned the role of p70 S6 kinase in 5'TOP messenger translation [37].

We investigated the regulation of protein synthesis and translation factors during the early phase of activation of resting T cells by αCD3 and αCD28, i.e. 1 h of activation. The protein synthesis rate in T cells rapidly increased after
treatment (Fig. 1), and several signalling pathways, i.e. ERK and p38 MAPK phosphorylation and PKB activation, were stimulated, showing the efficacy of the αCD3 and αCD28 antibodies in activating the cells.

The increase in protein synthesis after 1 h was mediated via multiple signalling pathways, e.g. the MEK, p38 MAPK/β, PI 3-kinase and mTOR pathway, as indicated by the use of signal transduction inhibitors (Fig. 3). In several cell types, the involvement of either MEK [40–44], PI 3-kinase [23,40,45,46] or mTOR [23,40,45–47] in the activation of protein synthesis has been described. However, this is the first time that a role for the p38 MAPKα/β pathway (Fig. 3) in the regulation of overall protein synthesis has been described. This role is supported by the fact that two structurally unrelated p38 MAPKα/β inhibitors, i.e., SB202190 and SB203580, were each able to block the increase in protein synthesis.

The increase in protein synthesis after activation of T cells with αCD3 and αCD28 coincided with an increase in the activities of p70 S6 kinase and eIF2B and dephosphorylation of eEF2, which is indicative of an increase in its activity [7]. The dephosphorylation of eEF2 (Fig. 6) was very transient, and therefore it is unlikely that eEF2 plays an important role in the sustained increase in protein synthesis after T cell activation. However, activation of eEF2 could be important for the initial increase in protein synthesis.

The stimulation of p70 S6 kinase was mediated via similar signalling pathways (Fig. 7) to those underlying the activation of overall protein synthesis. Inhibition of PI 3-kinase, mTOR, p38 MAPKα/β, or MEK during T cell activation by αCD3 and αCD28 prevented the activation of p70 S6 kinase, indicating that multiple signalling pathways are required for regulation of p70 S6 kinase activity. The effect of rapamycin on the activation of p70 S6 kinase in response to αCD3 and αCD28 has been reported previously [48]. Inhibition of the activation of p70 S6 kinase by SB203580 has been described before in insulin-stimulated rat vascular smooth muscle cells [43,49–51]. Furthermore, it has been reported that SB203580 (at the concentration used, 10 μM) can inhibit phosphorylation of PKB at Threonine308 and thus its activation [52,53]. Since PKB is an upstream component of the signalling pathway towards p70 S6 kinase, this could provide a mechanism by which SB203580 blocks activation of p70 S6 kinase.

Each of the other signalling pathways studied here has also been implicated in the regulation of p70 S6 kinase activity in a variety of cell types under a range of conditions [43,49–51]. However, in human primary T cells all of them appear to be important for the regulation of p70 S6 kinase activity after stimulation with αCD3 and αCD28.

The activity of the guanine nucleotide exchange factor, eIF2B, also was mediated via similar signalling pathways as the increase in protein synthesis (Fig. 5).

The increase in eIF2B activity after activation of T cells with αCD3 and αCD28, at the early times we examined, was not due to an increase in eIF2B protein level or to changes in eIF2α phosphorylation. Therefore, modification of the eIF2B protein complex probably caused the increase in eIF2B activity. The modulation of the activity of the eIF2B complex after activation of T cells with αCD3 and αCD28 required several different signalling pathways, e.g. MEK, p38 MAPKα/β, mTOR and the PI 3-kinase pathway. These signalling pathways have been reported separately to be involved in the regulation of eIF2B activity [16,23,42,44,54]. However, this is the first report where all these pathways are involved in the regulation of eIF2B in a single cell type.

A small inactivation of GSK-3 was detected after activation of primary T cells with αCD3 and αCD28. However, no dephosphorylation of Ser540 in eIF2B was detected (Fig. 5E), excluding a role for GSK-3 in regulating the activity of eIF2B under these conditions. In contrast, studies employing PMA/ionomycin-activated T cells [25], insulin treatment of various cell types [34], and cell survival [35] have implied a role for GSK-3 in regulating eIF2B activity.

In contrast to previously reported data using mitogenic stimuli to activate primary T cells or Jurkat T cells [14–17], eIF4E phosphorylation, association of eIF4G with eIF4E and 4E-BP1 phosphorylation remained unchanged after T cell activation using αCD3 and αCD28 as a stimulus (Fig. 4). Signal transduction inhibitor studies showed that the MEK and p38 MAPKα/β pathways are important for eIF4E phosphorylation in Jurkat T cells [17], and a role for MEK was demonstrated previously in primary T cells [16]. The weaker activation of the ERK pathway in particular (Fig. 1) by αCD3 and αCD28 in primary T cells may well account for the absence of increased phosphorylation of eIF4E under these conditions.

We did not observe increased phosphorylation of 4E-BP1 in response to αCD3 and αCD28, even though it has been reported to occur after cytokine stimulation of a murine cytotoxic T cell line [20] or after 6 h of mitogenic stimulation of human primary T cells [16]. However, Grolleau et al. [32] showed that 4E-BP1 was present in primary T cells mainly in its dephosphorylated form, and no significant change was detected after PMA treatment. Our results are consistent with this last finding: 4E-BP1 is mainly present as one band, and no change in mobility is observed upon
cell treatment. This is consistent with the observation that elf4F complex formation did not alter. However, it remains surprising that elf4F complexes are present when 4E-BP1 is completely dephosphorylated, and therefore presumably associated with elf4E. We were unable to detect any 4E-BP1 associated with elf4E, which is probably due to the low 4E-BP1 protein levels in resting T lymphocytes, thus explaining basal elf4F formation.

Conclusions
The treatment of primary T lymphocytes with αCD3 and αCD28 activates two key components of the translational machinery, p70 S6 kinase and eIF2B. The activities of these translation factors were regulated similarly to the activation of protein synthesis, consistent with an important role for the components in the activation of protein synthesis by αCD3 and αCD28. Interestingly, activation of protein synthesis, p70 S6 kinase, and elf2B is inhibited by rapamycin, a compound that was first discovered as an immunosuppressant, suggesting that mTOR regulated translation is involved in the process of T cell proliferation. The activation of p70 S6 kinase is related to the regulation of translation of specific mRNAs, while the activation of elf2B is likely to be required for stimulation of general protein synthesis [13]. This suggests that increases in both specific and general protein synthesis are important in the early phase of T cell activation.

Materials and methods
Primary T cell isolation and cell treatment
Buffy coats used for the isolation of T cells were prepared from freshly drawn blood from healthy human donors and were obtained from the Scottish National Blood Transfusion Service (Edinburgh, UK). Mononuclear leukocytes were isolated by ficoll-hypaque (Amersham-Pharmacia) gradient centrifugation. T cells were further enriched using nylon-wool columns. T cells were kept in 75 cm² tissue culture flasks at a density of 4 × 10⁶ cells/ml at 37°C and 5% CO₂. All tissue culture reagents were obtained from Gibco BRL.

Measurement of protein synthesis rate
Cells were treated with αCD3 mouse IgG2a mAb (33/2A3) (1:1000 dilution from a hybridoma supernatant) and αCD28 mouse IgM mAb (CK243) (1:12 dilution from a hybridoma supernatant) for 1 h in the absence or presence of signal transduction inhibitors. For the last 30 min, 10 µCi/ml [³⁵S]methionine was added to the cells. To harvest the cells, the cells were transferred to a microfuge tube and centrifuged at 6000 × g for 20 s. The cell pellet was lysed in 20 mM Hepes pH 7.4, 50 mM β-glyceroophosphate, 0.2 mM EDTA, 1% Triton X-100, 10% (v/v) glycerol, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml antipain, 1 mM benzamidine, and 1 mM DTT. Part of the sample was used to measure the protein content with Protein Assay Reagent (Bio-Rad) and the rest was spotted in duplicate on to Whatman 3 MM paper and subjected to 'hot TCA precipitation'.

Gel electrophoresis and Western blotting
T cells were activated with αCD3 and αCD28 for times indicated in the figure legends, harvested in Laemmli sample buffer and analyzed by SDS-PAGE and Western blotting. Phospho-p42/44 (ERK) and phospho-p38 MAPK antibodies were obtained from New England Biolabs, the phospho-elf2α antibody was a kind gift from Dr. Gary Krause (Detroit, USA), the phospho-S6 (Ser235) antibody was a kind gift from Dr. Dario Alessi (University of Dundee), the 4E-BP1 antibody was obtained from Santa Cruz (SC-6025), the elf2Be antibody was raised in rabbit against the whole protein expressed in the baculovirus system [44], the phospho-specific antibody for Ser540 in elf2Be was raised against the peptide SEEPDSP(R)RGGC (S(P) indicates the phosphoserine) in sheep, and the phospho-elf2β (Thr56) antibody was raised against the peptide GETRTF(P)DTRK (T(P) indicates phosphothreonine) [55].

Kinase assays
For PKB assays, the cells were pelleted at 6000 × g for 20 s and harvested in 50 mM Tris-HCl pH 7.5, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 50 mM NaF, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 µM microystin LR, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml antipain, and 1 mM benzamide-HCl. Antibodies directed against the three PKB isoforms (α, β, and γ) were simultaneously bound to protein G-Sepharose, and about 100 µg of protein was used in immunoprecipitation reactions. The immunoprecipitation and PKB assays were performed as described before [56].

For p70 S6 kinase and GSK-3 assays, the cells were harvested in a buffer containing 50 mM Tris-HCl pH 7.5, 50 mM β-glycerophosphate, 0.5 mM sodium vanadate, 1.5 mM EDTA, 1.5 mM EGTA, 0.5% Triton X-100, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml antipain, 1 mM benzamidine, and 1 mM DTT. A polyclonal antibody raised against a peptide sequence from p70 S6 kinase-1 was bound to protein G-Sepharose, and about 100 µg of extract was used in the immunoprecipitation reaction. Immunoprecipitation and the p70 S6 kinase assays (using a peptide substrate) were performed as described before [57]. GSK-3α and β were immunoprecipitated together from 150 µg of cell lysate and kinase assays were performed as described [23,58].
Phosphorylation of eIF4E and eIF4F complex formation

Cells were pelleted at 6000 x g for 20 s and harvested in a buffer containing 20 mM Hepes pH 7.4, 50 mM β-glycerophosphate, 0.2 mM EDTA, 1% Triton X-100, 10% (v/v) glycerol, 1 µg/ml leupeptin, 1 µg/ml peptatin, 1 µg/ml antipain, 1 mM benzamidine, and 1 mM DTT. Using m7GTP Sepharose 4B (Amersham-Pharmacia; 15 µl of slurry diluted with 15 µl of Sepharose CL-4B), eIF4E was purified from approximately 2 mg of extract. For SDS-PAGE, Laemmli sample buffer was added and the samples were heated at 95°C for 10 min. For one-dimensional iso-electric focusing analysis, the appropriate sample buffer was added [59]. The samples were run on a 12.5% SDS-PAGE gel or on a one-dimensional iso-electric focusing gel, transferred to PVDF, and detected by Western analysis. eIF4E was detected with a polyclonal antibody raised in rabbit [43], and eIF4GI with a polyclonal antibody raised in sheep against the peptide CKKEAVGDLLDAFKEAN.

Measurement of eIF2B activity

The cells were pelleted at 6000 x g for 20 s and lysed in a buffer containing 20 mM Tris-HCl pH 7.5, 50 mM β-glycerophosphate, 100 mM KCl, 0.2 mM sodium orthovanadate, 0.2 mM EDTA, 0.2 mM EGTA, 1% Triton X-100, 10% glycerol, 1 µg/ml leupeptin, 1 µg/ml peptatin, 1 µg/ml antipain, 1 mM benzamidine, and 1 mM DTT. About 50 µg of cell lysate was used for the eIF2B assay, which was performed as described previously [23].

Authors’ contributions

Author 1 (MK) carried out all the experiments. Author 2 (CPG) participated in the design and coordination of this study. All authors read and approved the final manuscript.

Abbreviations

eIF, eukaryotic initiation factor; eIF, eukaryotic elongation factor; ERK, extracellular regulated kinase; GSK-3, glycogen synthase kinase-3; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; PI 3-kinase, phosphoinositide 3-kinase; PHA, phytohemagglutinin; PKB, protein kinase B; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; 4E-BP1, eIF4E-binding protein 1.

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