Insulin and IGF-I stimulate the formation of the eukaryotic initiation factor 4F complex and protein synthesis in C2C12 myotubes independent of availability of external amino acids

Wei-Hua Shen, David W Boyle, Paul Wisniowski, Aashia Bade and **Edward A Liechty**

Herman B Wells Center for Pediatric Research, Department of Pediatrics, Indiana University School of Medicine, Indianapolis, Indiana, USA
(Requests for offprints should be addressed to E A Liechty, Riley R208, 699 West Drive, Indianapolis, IN 46202, USA; Email: eliecht@iupui.edu)

Abstract

The objective of this study was to investigate the effect of insulin and IGF-I on protein synthesis and translation initiation in C2C12 myotubes in nutrient-deprived Dulbecco's phosphate buffered saline (DPBS). The results showed that insulin and IGF-I increased protein synthesis by 62% and 35% respectively in DPBS, and the effect was not affected by rapamycin, but was blocked by LY294002. Insulin and IGF-I stimulated eukaryotic initiation factor 4E (eIF4E) binding protein (4EBP1) phosphorylation in a dose-dependent manner, and the stimulation was independent of availability of external amino acids. Both LY294002 and rapamycin blocked the insulin and IGF-I-induced increases in 4EBP1 phosphorylation. The results also showed that insulin and IGF-I were able to stimulate PKB/Akt phosphorylation, glycogen synthase kinase (GSK) 3β phosphorylation and mTOR phosphorylation in DPBS. Insulin and IGF-I increased the amount of eIF4G associated with eIF4E in nutrient-deprived C2C12 myotubes. The amount of 4EBP1 associated with eIF4E was decreased after insulin or IGF-I stimulation. We conclude that in C2C12 myotubes, insulin and IGF-I may regulate protein synthesis and translation initiation independent of external amino acid supply via the phosphatidylinositol-3 kinase-PKB/Akt-mTOR pathway.

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Introduction

Initiation of mRNA translation is a complex process requiring several steps and more than a dozen eukaryotic initiation factor (eIFs) (Pain 1996). It is proposed that the binding of mRNA to the 43S preinitiation complex is one of the rate-limiting steps in translation initiation. The binding of mRNA to the 43S preinitiation complex is regulated by a group of eIFs collectively called eukaryotic initiation factor 4F (eIF4F). eIF4F is a multi-subunit complex consisting of three components: eIF4A, eIF4E, and eIF4G. The eIF4F complex serves to recognize, unfold and guide the mRNA to the 43S preinitiation complex.

Formation of a translationally active eIF4F complex may be regulated by alterations in either phosphorylation state or availability of eIF4E. Phosphorylation of eIF4E is suggested to stimulate translation rates through increased association with eIF4G and eIF4A (Bu *et al.* 1993) and/or increased mRNA cap binding affinity (Minich *et al.* 1994). However, recent studies have suggested that our understanding of the role of eIF4E phosphorylation in translational control is far from complete, and the functional consequences of eIF4E phosphorylation remain controversial (Morley & Naegele 2002). eIF4E is the least abundant of the eIF4F subunits, and it is generally believed that the amount of eIF4E is limiting for translation initiation. Availability of eIF4E to form the eIF4F complex appears to be regulated by a group of small acid- and heat-stable proteins termed eIF4E binding proteins (4EBPs): 4EBP1 (Pause *et al.* 1994), 4EBP2 (Pause *et al.* 1994) and 4EBP3 (Poulin *et al.* 1998). Of these, the regulation of 4EBP1 has been described in most detail. 4EBP1 competes with eIF4G for binding to eIF4E. Since 4EBP1 and eIF4G interact with overlapping binding sites on eIF4E, their binding is mutually exclusive (Haghihat *et al.* 1995). The binding of eIF4E with 4EBP1 is regulated by 4EBP1 phosphorylation. Hypophosphorylated 4EBP1 binds tightly to eIF4E to form the translationally inactive eIF4E–4EBP1 complex. Upon 4EBP1 phosphorylation, eIF4E is released from the translationally inactive eIF4E–4EBP1 complex and is presumably available for binding to eIF4G, and through eIF4G to the 40S ribosome.

It has been demonstrated that insulin and other growth factors acutely stimulate protein synthesis by regulating translation initiation (Shah *et al.* 2000*a*). In addition,

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nutrients, especially amino acids, also play a role in controlling the activity and/or activation of certain proteins involved in regulating mRNA translation (Hara *et al.* 1998, Kimball *et al.* 1998*a*, 1999, Patti *et al.* 1998). Molecular mechanisms of the regulation of translation initiation induced by growth factors and amino acids appear to be clear. Insulin and insulin-like growth factor I (IGF-I) stimulate 4EBP1 phosphorylation via the phosphatidylinositol-3 kinase (PI-3 kinase)-mTOR pathway (Graves *et al.* 1995, Mendez *et al.* 1996, Scott *et al.* 1998), whereas amino acids stimulate 4EBP1 phosphorylation in a PKB/Akt-independent manner, but dependent on mTOR (Hara *et al.* 1998, Xu *et al.* 1998).

There are inconsistent reports concerning whether the growth factor-induced alterations in translation initiation require the presence of external amino acids. An increasing body of evidence has shown that the insulin-, IGF-I- and other growth factor-induced alterations in translation initiation such as 4EBP1 phosphorylation and the formation of the eIF4F complex are mediated by amino acid availability (Hara *et al.* 1998, Xu *et al.* 1998, Campbell *et al.* 1999, Kleijn & Proud 2000, Dennis *et al.* 2001, Herbert *et al.* 2002). The addition of amino acids to amino acid-deprived cells only partially restores 4EBP1 phosphorylation and synergizes with insulin, IGF-I or serum to elicit complete phosphorylation (Hara *et al.* 1998, Xu *et al.* 1998).

The regulation of translation initiation by insulin and amino acids has been studied extensively in L6 myoblasts in culture (Kimball *et al.* 1998*b*, 1999, Shah *et al.* 2000*b*). The response of differentiated myotubes to growth factor stimuli and amino acids may be different from those of pre-differentiated myoblasts. Treating pre-differentiated myoblasts with growth factors results in an increase in proliferation (Svegliati-Baroni *et al.* 1999). In contrast, treating differentiated myotubes with growth factors results in hypertropy (Rommel *et al.* 1999), demonstrating the need to distinguish between pre-differentiation and post-differentiation effects. In the present study, C2C12 myotubes were used to investigate the regulation of translation initiation by insulin and IGF-I independent of amino acids. Our results showed that insulin and IGF-I stimulated protein synthesis and regulated translation initiation in nutrient-deprived C2C12 myotubes. Insulin and IGF-I regulated 4EBP1 phosphorylation, eIF4E-4EBP1 binding and eIF4E-eIF4G binding. These effects were mediated by the PI-3 kinase pathway; however, the mTOR protein was required for some but not all of these regulations.

Materials and Methods

Cell culture

The C2C12 mouse muscle cell line was from the American Type Culture Collection (ATCC, Manassas, VA, USA). C2C12 mouse myoblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) (high glucose with L-glutamine and pyridoxine hydrochloride) (Invitrogen Corporation) plus 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. For differentiation, when the cells were grown to ~80% confluence, they were then cultured in differentiation medium (DM) (DMEM, high glucose with L-glutamine and pyridoxine hydrochloride, plus 1% horse serum). The cells were characterized as myotubes by morphologic criteria. On day 6 of differentiation, the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS) and then placed in fresh DM or DPBS for 3 h. Then, cells were treated with porcine insulin (Eli Lilly and Company, Indianapolis, IN, USA) or IGF-I (Genentech, Inc., South San Francisco, CA, USA) for 40 min. Where indicated, rapamycin (Sigma, St Louis, MO, USA) or LY294002 (Sigma) was added 20 min before the insulin or IGF-I stimulation. The cells were then harvested in lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris (pH 7·4), 0·25% sodium deoxycholate, 2 mM EGTA, 1 mM EDTA, 0·2 mM sodium orthovanadate, 20 mM NaF, 1% proteinase inhibitor cocktail (Sigma) and 1% phosphatase inhibitor cocktail I and II (Sigma)), and centrifuged. Total proteins in the supernatant were measured by the Lowry method (Lowry *et al.* 1951). The supernatant was stored at –70 °C until further analysis.

Western blotting

The amount of initiation factors (eIF4E, eIF4G and 4EBP1) and signal proteins (PKB/Akt, mTOR) was measured by SDS-PAGE and Western blotting. The supernatant was diluted with SDS buffer, and subjected to reduced discontinuous SDS-PAGE at 90 V. An equal amount of protein (25 μg/lane) was loaded into each well. The proteins were transferred onto PVDF membranes with a BioRad transfer unit at 90 V for 90 min, and blocked with the blocking buffer (PBS containing 0·1% Tween 20 and 5% Carnation nonfat milk) for 1 h. The membrane was then incubated with primary antibodies (polyclonal antibody to eIF4G and mouse monoclonal antibody to eIF4E from Santa Cruz Biotechnology, Santa Cruz, CA, USA; the others from Cell Signaling Technology, Inc., Beverly, MA, USA) at 4 °C overnight. After washing with PBST (PBS+0·1% Tween 20), the membrane was then incubated with secondary antibodies (Santa Cruz Biotechnology) at room temperature for 1 h. After washing, the membrane was developed using an ECL Western blotting kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membrane was stripped and reprobed with a polyclonal antibody to actin (Santa Cruz Biotechnology) and/or stained with Coomassie Blue R-250 to confirm equal protein loading per sample.

Phosphorylation of eIF4E, eIF4G, 4EBP1 and the signal proteins (PKB/Akt, mTOR, ERK1/2 MAP kinase

(MAPK), Mnk1, p70S6K and glycogen synthase kinase 3β (GSK3β)) at designated sites was measured in the same way except that the phospho-specific antibodies (Cell Signaling Technology, Inc.) were used.

4EBP1 phosphorylation can also be determined by mobility on SDS-PAGE. Previous experiments have established that phosphorylation of 4EBP1 retards the protein migration rate on SDS-polyacrylamide gels (Lin *et al.* 1995, Kimball *et al.* 1996). Consequently, when the tissue or cell extract is subjected to 15% SDS-PAGE, multiple electrophoretic forms may be resolved. These forms have been identified as α (least phosphorylated and fastest migrating), β (intermediate), and γ (most phosphorylated and slowest migrating).

Films were scanned using ScanJet ADF (HP Intelligent Scanning Technology, Palo Alto, CA, USA), and the images were quantitated using the Scion image software (Scion Corporation, Frederick, MD, USA).

Determination of eIF4G or 4EBP1 associated with eIF4E

Association of 4EBP1 or eIF4G with eIF4E was assessed by determining how much eIF4G or 4EBP1 was recovered when eIF4E was extracted with m^7GTP Sepharose 4B (Amersham Pharmacia Biotech). The supernatant of an equal protein amount (250 μg/sample) was added to a microcentrifuge tube containing 80 μl of the Sepharose slurry, incubated for 1 h at 4 °C. The slurry was then washed three times with ice-cold lysis buffer (1 ml/wash). Finally, the slurry was then resuspended in 80 μl 2 × SDS sampling buffer and subjected to electrophoresis. Proteins were electrophoretically transferred to a PVDF membrane, and eIF4E, eIF4G and 4EBP1 were quantified by Western blotting as described above. Our preliminary study showed that the percentage recovery of eIF4E in this assay was 90%. The amount of eIF4G and 4EBP1 detected in the bound fraction represents eIF4G or 4EBP1 associated with eIF4E in the samples.

Protein synthesis

On day 6 of differentiation, cells were first cultured in DPBS for 1 h. Then, the cells were treated with insulin or IGF-I as described above. Where indicated, rapamycin, LY294002 or the carrier (DSMO) were added 20 min before the stimulation. Forty minutes after introducing insulin or IGF-I into the medium, [^{3}H]phenylalanine (American Radiolabeled Chemicals, Inc., St Louis, MO, USA) was added and the cells were maintained for another 120 min. The total time that the cells were maintained in DPBS in the study was 4 h, which was the same as that in the preceding eIF studies. The cells were washed with PBS twice and harvested in the lysis buffer. The protein in the supernatant was precipitated with cold 8% perchloric acid (PCA), and the pellets were resuspended in 8% PCA and washed three times. The protein synthetic rate was determined by measuring the incorporation of radioactivity into the cell proteins.

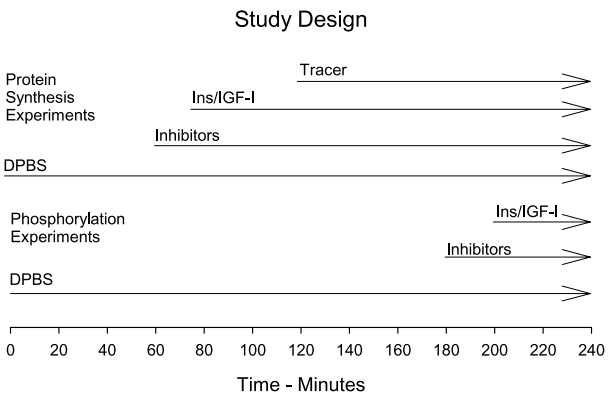


Figure 1 Study design utilized in the experiments. The time in minutes for culturing cells in Dulbecco's phosphate buffered saline (DPBS) and the time in minutes that specific substrates (tracers), hormones (insulin (Ins) or IGF-I), or inhibitors (LY294002 or rapamycin) were added to the culture medium are depicted for both the protein synthesis experiments and the eIF protein phosphorylation experiments.

The study design timeline is shown in Fig. 1.

Reproducibility and statistics

All experiments were performed three or four times, with similar results. Blots were scanned, and densitometry analysis was performed. All density data were normalized to control on the same blot. The data were analyzed either by one-way ANOVA, followed by Tukey's Honestly Significant Difference (HSD) test (JMP, SAS Institute, Cary, NC, USA) if a significant overall effect was detected, or simply by Student's *t*-test (Microsoft Excel) when two groups were compared. The results are presented as means ± S.E.M. of three experiments. Representative blots are also given above the graphs.

Results

Insulin (200 nM) and IGF-I (130 nM) increased protein synthesis by 62% and 35% respectively in nutrient-deprived C2C12 myotubes (Fig. 2). However, no additive effect was found when the two peptides were used together. Addition of LY294002 or rapamycin into the medium significantly decreased basal protein synthesis in nutrient-deprived C2C12 myotubes. The insulin- and IGF-I-stimulated protein synthesis was blocked by LY294002 (32 μM) ($P<0{\cdot}05$), but was not affected by rapamycin (128–1280 nM). It is of interest that additive inhibition was found when the two inhibitors were used together. However, it is apparent that, in the presence of rapamycin or LY294002, insulin and IGF-I were still able to increase global protein synthesis.

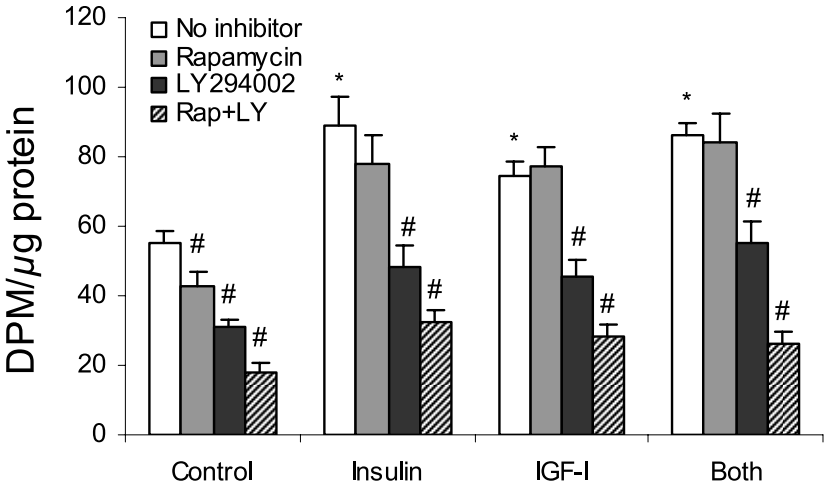


Figure 2 Effects of rapamycin and LY294002 on stimulation of protein synthesis by insulin, IGF-I or both in nutrient-deprived C2C12 myotubes. Cells were first maintained in DPBS for 1 h on day 6 of differentiation, and then treated with insulin, IGF-I or both. Where indicated, rapamycin (Rap, 128 nM) or LY294002 (LY, 32 μM) or both were added 20 min before the insulin or IGF-I stimulation. Forty minutes after introducing insulin or IGF-I into the medium, [^{3}H]phenylalanine was then added and the cells were maintained for another 120 min. The cells were washed with PBS twice and harvested in lysis buffer. The protein was precipitated with cold 8% perchloric acid (PCA), and the pellets were resuspended in PCA and washed three times. The protein synthetic rate was determined by measuring radioactivity incorporation into the cell proteins (DPM/μg protein). The data are means ± S.E.M. of three separate cell preparations. *P<0·05 relative to control without inhibitors; #P<0·05 relative to corresponding values without inhibitors (i.e. compared with the control alone, insulin alone, IGF-I alone or insulin+IGF-I alone group respectively).

C2C12 cells in nutrient-deprived DPBS exhibited decreased 4EBP1 phosphorylation compared with those in differentiation medium, suggesting a role of nutrients in 4EBP1 phosphorylation (Fig. 3A). However, in both differentiation medium and nutrient-deprived DPBS, insulin and IGF-I were able to stimulate 4EBP1 phosphorylation (Fig. 3A). The stimulation in nutrient-deprived DPBS was dose dependent (Fig. 3B). Since a physiological dose of insulin (~0·2 nM) and free IGF-I (~0·13 nM) were unable markedly to increase 4EBP1 phosphorylation in the nutrient-deprived cells, a pharmacological dose (1000-fold of physiological dose) of insulin (200 nM) or IGF-I (130 nM) was used in the other experiments. The findings suggest that, in C2C12 myotubes, insulin and IGF-I can stimulate 4EBP1 phosphorylation independent of extracellular amino acid supply.

Previous studies suggest that insulin and IGF-I stimulate 4EBP1 phosphorylation via the PI-3 kinase-mTOR pathway. In the present studies, two specific kinase inhibitors, rapamycin (an mTOR inhibitor) and LY294002 (a PI-3 kinase inhibitor), were used to determine if this same regulatory mechanism applied to the insulin- and IGF-I-stimulated 4EBP1 phosphorylation in C2C12 myotubes. The concentrations of the inhibitors used in this study were previously demonstrated to effectively block the designated kinase activity (Wang *et al.* 1998, Nave *et al.* 1999). Our results showed that the basal level of 4EBP1 phosphorylation was not affected by either rapamycin or LY294002 (data not shown). However, the insulin- and IGF-I-induced increase in 4EBP1 phosphorylation was blocked by LY294002 and rapamycin (Fig. 4A,B).

It has previously been demonstrated that multiple Ser/Thr residues of 4EBP1 are phosphorylated in a hierarchical pattern. Six phosphorylation sites have been identified in 4EBP1 - Thr37, Thr46, Ser65, Thr70, Ser83, and Ser112. In the present studies, 4EBP1 phosphorylated at Thr70 was measured using a phospho-specific antibody. As shown in Fig. 4C and D, the antibody to 4EBP1-Thr70 only recognized the β and γ forms of 4EBP1. The total amount of 4EBP1 phosphorylated at Thr70 was not changed significantly after insulin or IGF-I stimulation, but it is apparent that the two peptides were able to stimulate overall 4EBP1 phosphorylation, again based on changes in relative distribution of the two highly phosphorylated 4EBP1 forms (β and γ), and the stimulation was attenuated by rapamycin and blocked by LY294002. These data indicate that both rapamycin-sensitive and LY294002-sensitive pathways are required for the stimulation of 4EBP1 by insulin or IGF-I.

In the same study, p70S6K, a well established downstream effector of PI-3 kinase and mTOR, was also activated by insulin and IGF-I, indicated by increased

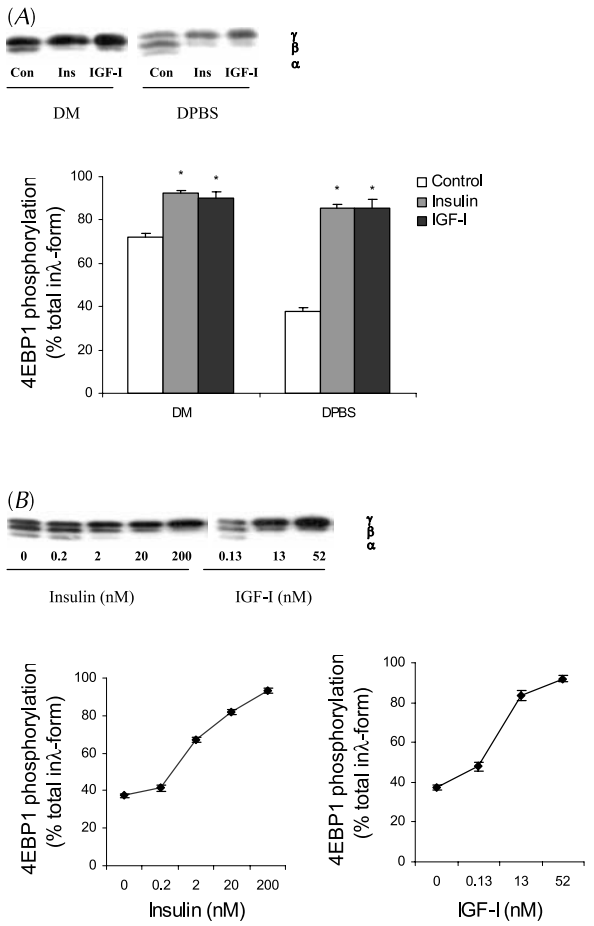


Figure 3 Insulin (Ins, 200 nM) and IGF-I (130 nM) stimulated 4EBP1 phosphorylation in C2C12 myotubes in differentiation medium (DM) and in Dulbecco's phosphate buffered saline (DBPS) (A); insulin- and IGF-I-induced 4EBP1 phosphorylation in nutrient-deprived C2C12 myotubes was dose-dependent (B). Cells were first maintained in DM or DPBS for 3 h on day 6 of differentiation, and then treated with insulin or IGF-I for 40 min. Increased 4EBP1 phosphorylation following insulin or IGF-I stimulation was manifested by a retardation of its mobility on 15% SDS-PAGE, and expressed as a percentage of total in the γ-form. *P<0·05 relative to corresponding values of control. Con, control; α, least phosphorylated and fastest migrating form of 4EBP1; β, intermediate form of 4EBP1; γ, most phosphorylated and slowest migrating form of 4EBP1.

phosphorylation at Thr389/Thr421/Ser424 (Fig. 5). The stimulation was blocked by rapamycin.

As shown in Fig. 6, insulin and IGF-I caused decreased 4EBP1 associated with eIF4E. In a reciprocal manner, insulin and IGF-I increased the amount of eIF4G associated with eIF4E, indicative of increased levels of the eIF4F complex. The insulin- and IGF-I-induced alterations in eIF4E-4EBP1 binding were attenuated or blocked by rapamycin and LY294002, but LY294002 exhibited much more potent inhibition than rapamycin. Rapamycin increased association of 4EBP1 in the α form with eIF4E, whereas LY294002 increased association of 4EBP1 in both the α and β forms with eIF4E (Fig. 6). Regarding eIF4E-eIF4G binding, rapamycin had no effect on the insulin- and IGF-I-induced increase in eIF4E-eIF4G binding (P>0·05). However, LY294002 significantly blocked the insulin- and IGF-I-induced increases in eIF4E-eIF4G binding in nutrient-deprived C2C12 myotubes (P<0·05) (Fig. 6).

The basal level of eIF4E-4EBP1 binding was not affected by either rapamycin or LY294002 (Fig. 6). The basal level of eIF4E-eIF4G binding was not affected by rapamycin, but it was markedly reduced by LY294002 treatment (P<0·05) (Fig. 6).

It has been suggested that, in addition to 4EBP1 phosphorylation, eIF4E phosphorylation and eIF4G phosphorylation may also be involved in the regulation of the formation of the eIF4F complex and/or protein synthesis. As shown in Fig. 7A, eIF4E phosphorylation was not altered following insulin or IGF-I treatment. Since eIF4E phosphorylation may change in a cyclic manner, a time course study of insulin stimulation was conducted. As shown in Fig. 7B-D, eIF4E phosphorylation was not altered within 60 min after the stimulation, although the phosphorylation of p42/44 MAPK, indicative of activation of the MAPK pathway, was elevated between 5 and 15 min after the insulin stimulation. Mnk1, downstream of MAPK but upstream of eIF4E, also exhibited a transient activation, indicated by increased phosphorylation at Thr197/202.

Insulin and IGF-I stimulated eIF4G phosphorylation at Ser1108, and the effects were all attenuated or blocked by rapamycin or LY294002 (Fig. 8). However, the basal level of the eIF4G phosphorylation was not altered by the two kinase inhibitors.

Using the specific kinase inhibitors, LY294002 and rapamycin, our preceding results have suggested that insulin and IGF-I may stimulate 4EBP1 phosphorylation via the PI-3 kinase-mTOR pathway. To further assess the role of this pathway, we measured phosphorylation of PKB/Akt and mTOR, two indicative proteins in this pathway. The phosphorylation of the two kinases at specific sites is necessary to render the kinases active. Our results showed that insulin and IGF-I stimulated PKB/Akt phosphorylation at Ser473 in a dose-dependent manner and the stimulation was blocked by LY294002 (Fig. 9). mTOR phosphorylation at Ser2448 was also enhanced by insulin and IGF-I, and the effect was blocked by LY294002, but not by rapamycin (Fig. 10). The results further confirmed that insulin and IGF-I stimulate 4EBP1 phosphorylation via the PI-3 kinase-PKB/Akt-mTOR pathway.

In addition to mTOR, GSK3 represents another downstream element of the PI-3 kinase-PKB/Akt pathway, and its activity can be inhibited by PKB/Akt-mediated phosphorylation. As shown in Fig. 11, insulin and IGF-I increased GSK3β phosphorylation in nutrient-deprived

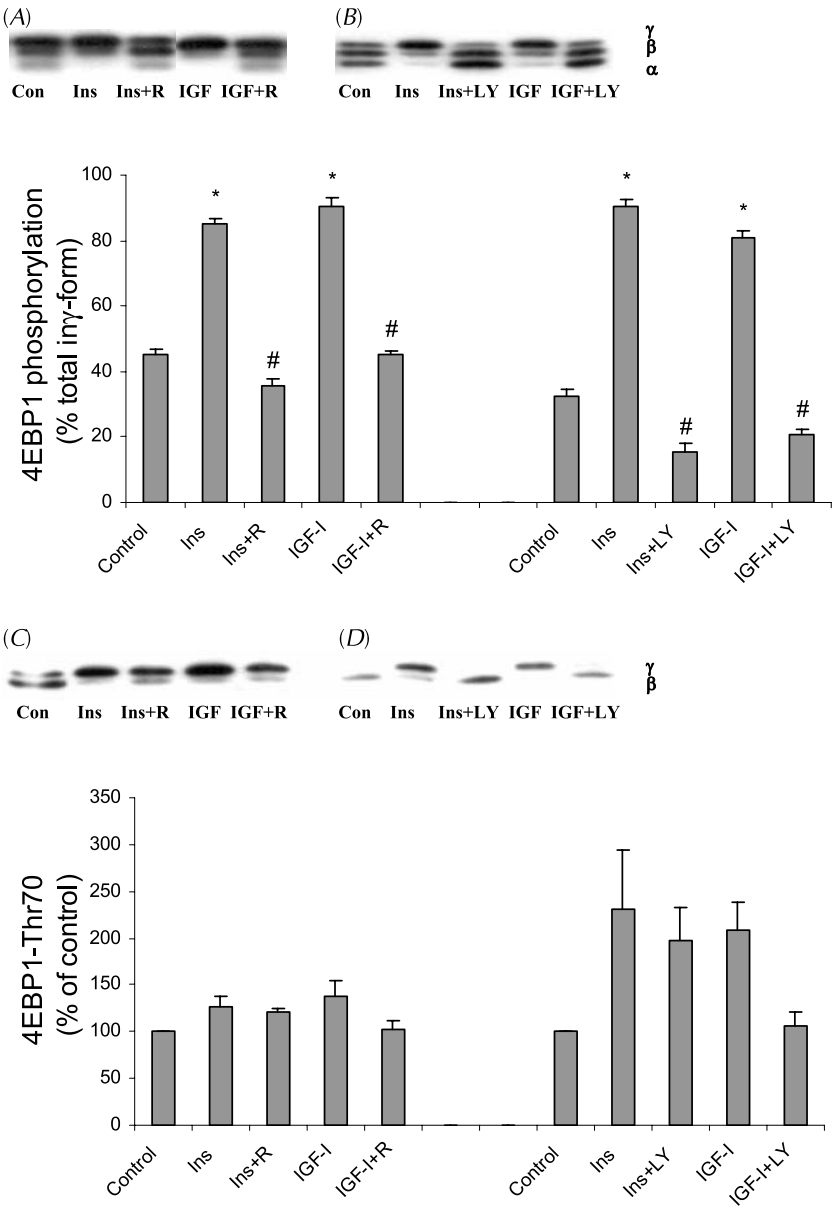


Figure 4 Insulin- (Ins, 200 nM) and IGF-I (IGF, 130 nM)-induced increase in 4EBP1 phosphorylation in C2C12 myotubes was largely blocked by an mTOR inhibitor, rapamycin (R, 128 nM) (A and C), and was completely blocked by a PI-3 kinase inhibitor, LY294002 (LY, 32 μM) (B and D). Cells were first maintained in DPBS for 3 h on day 6 of differentiation, and then treated with insulin or IGF-I for 40 min. Where indicated, rapamycin or LY294002 was added 20 min before insulin or IGF-I stimulation. Increased 4EBP1 phosphorylation following insulin or IGF-I stimulation was manifested by a retardation of its mobility on 15% SDS-PAGE either using a pan-4EBP1 antibody (A and B), or a phospho-specific antibody (4EBP1-Thr70) (C and D). 4EBP1 phosphorylation was expressed as a percentage of total in the γ-form. Phospho-4EBP1 at Thr70 was expressed as a percentage of control. *P<0·05 relative to corresponding value of control; #P<0·05 relative to corresponding values without the inhibitors (i.e. the insulin alone or IGF-I alone group). Con, control; α, least phosphorylated and fastest migrating form of 4EBP1; β, intermediate form of 4EBP1; γ, most phosphorylated and slowest migrating form of 4EBP1.

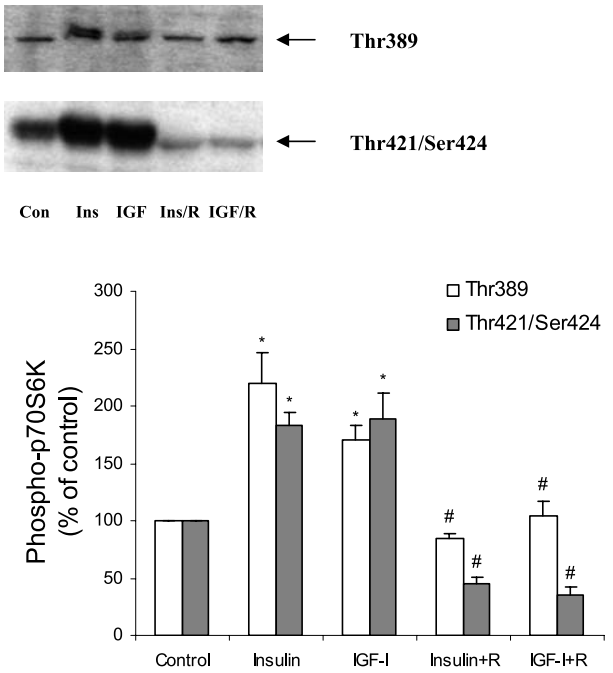


Figure 5 Insulin- (Ins, 200 nM) and IGF-I (IGF, 130 nM)-induced increase in p70S6K phosphorylation in C2C12 myotubes was largely blocked by an mTOR inhibitor, rapamycin (R, 128 nM). Cells were first maintained in DPBS for 3 h on day 6 of differentiation, and then treated with insulin or IGF-I for 40 min. Where indicated, rapamycin was added 20 min before insulin or IGF-I stimulation. Alterations in p70S6K phosphorylation were expressed as a percentage of control (con). *$P<0{\cdot}05$ relative to corresponding values of control; #$P<0{\cdot}05$ relative to corresponding values without inhibitors (i.e. the insulin alone or IGF-I alone group).

C2C12 myotubes, and the effect was blocked by LY294002. However, insulin- and IGF-I-induced GSK3β phosphorylation was not influenced by rapamycin (data not shown).

Discussion

Insulin and IGF-I stimulated 4EBP1 phosphorylation in nutrient-deprived C2C12 myotubes

The present results showed that insulin and IGF-I were able to stimulate 4EBP1 phosphorylation in both DM and nutrient-deprived C2C12 myotubes (Fig. 3), suggesting that, in C2C12 myotubes, insulin and IGF-I may stimulate 4EBP1 phosphorylation independent of the availability of external amino acids. Our results are consistent with those in FAO hepatocytes and rat epididymal fat cells (Diggle *et al.* 1996, Patti *et al.* 1998). However, our results are in contrast to many other studies, where 4EBP1 phosphorylation decreases when amino acids are withdrawn from cells, and insulin and other growth factors are unable to activate 4EBP1 phosphorylation under these conditions (Hara *et al.* 1998, Xu *et al.* 1998, Campbell *et al.* 1999, Kleijn & Proud 2000, Dennis *et al.* 2001, Beugnet *et al.* 2003). It is unclear why there is this inconsistency, but it seems apparent that the insulin-induced 4EBP1 phosphorylation may or may not require the presence of external amino acids, depending on cell type.

It has recently been demonstrated that it is intracellular amino acids, rather than extracellular ones, that are required for insulin and other growth factors to stimulate 4EBP1/p70S6K phosphorylation (Beugnet *et al.* 2003). Some cell types appear to contain sufficient intracellular amino acids for growth factors to regulate 4EBP1 phosphorylation even when starved of external amino acids, while in other cell types where autophagy is less active and/or protein synthesis is very active and intracellular amino acids are rapidly depleted, the stimulation of 4EBP1 phosphorylation may be more dependent on external amino acid supply.

In the present study, prior to the stimulation, C2C12 myotubes had been placed in DPBS for 3 h; however, intracellular amino acid concentrations in cells maintained in DPBS were only 10–15% lower than those maintained in differentiation medium (data not shown). This is in contrast to the findings in other studies where intracellular amino acid concentrations were only 22% of the control after cells were starved of external amino acids for 1 h (Beugnet *et al.* 2003). Our results suggest that C2C12 myotubes may be able to retain a high level of intracellular amino acids even when starved of external amino acids. This may help explain why insulin and IGF-I were able to stimulate 4EBP1 phosphorylation in C2C12 myotubes maintained in DPBS for 3 h.

We have not fully investigated if additional supplies of external amino acids and/or glucose may exhibit an additive effect on the insulin-induced 4EBP1 phosphorylation in C2C12 myotubes. However, we favor the hypothesis that the hormonal signal and nutrient signal are likely to be activated by different mechanisms and therefore exhibit additive or synergistic effects in regulating translation initiation. The two signal inputs may act on different phosphorylation sites of 4EBP1, and phosphorylation of certain sites may act as priming events of the ordered phosphorylation of 4EBP1.

Insulin and IGF-I increased eIF4G associated with eIF4E, and decreased 4EBP1 associated with eIF4E in nutrient-deprived C2C12 myotubes

Previous studies have demonstrated that, like 4EBP1 phosphorylation, eIF4G associated with eIF4E or 4EBP1 associated with eIF4E was not affected by insulin treatment in the absence of external amino acids, suggesting that the regulation of the eIF4F complex again requires the presence of external amino acids (Campbell *et al.* 1999, Beugnet *et al.* 2003). In contrast, we found that both

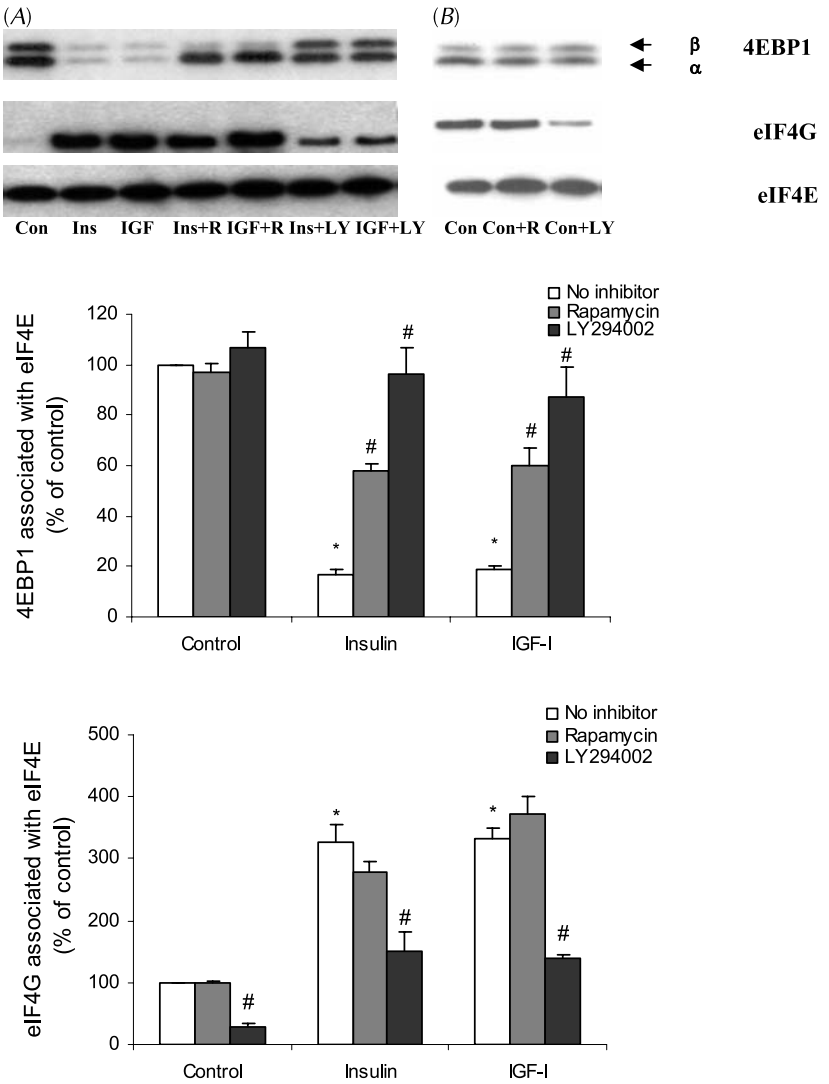


Figure 6 Effects of insulin (Ins, 200 nM) and IGF-I (IGF, 130 nM) on eIF4E-4EBP1 association and eIF4E-eIF4G association, and inhibition of the effect of either hormone by rapamycin (R, 128 nM) or LY294002 (LY, 32 μM) (A). Cells were first maintained in DPBS for 3 h on day 6 of differentiation, and then treated with insulin or IGF-I for 40 min. Where indicated, LY294002 or rapamycin was added 20 min before insulin or IGF-I stimulation. eIF4E in the cell lysate was extracted using m^7GTP Sepharose 4B. The m^7GTP Sepharose 4B-bound fraction was analyzed by SDS-PAGE and Western blotting using the antibody for 4EBP1, eIF4G or eIF4E. The data are means ± S.E.M. of three separate cell preparations, determined by densitometric scanning. The results were normalized to the amount of eIF4E. **P*<0·05 relative to corresponding values of control without inhibitors; #*P*<0·05 relative to corresponding values without the inhibitors (i.e. compared with the control alone, insulin alone, or IGF-I alone group respectively). Con, control; α, least phosphorylated and fastest migrating form of 4EBP1; β, intermediate phosphorylated form of 4EBP1.

insulin and IGF-I decreased the amount of eIF4E associated with 4EBP1 and increased eIF4E associated with eIF4G in C2C12 myotubes in DPBS (Fig. 6).

Rapamycin only partially prevented the insulin- and IGF-I-induced decrease in the amount of eIF4E associated with 4EBP1 (Fig. 6), although it completely blocked the insulin- and IGF-I-induced increase in 4EBP1 phosphorylation (Fig. 4). However, LY294002 completely blocked the insulin- and IGF-I induced decrease in the amount of eIF4E associated with 4EBP1. The findings suggest that

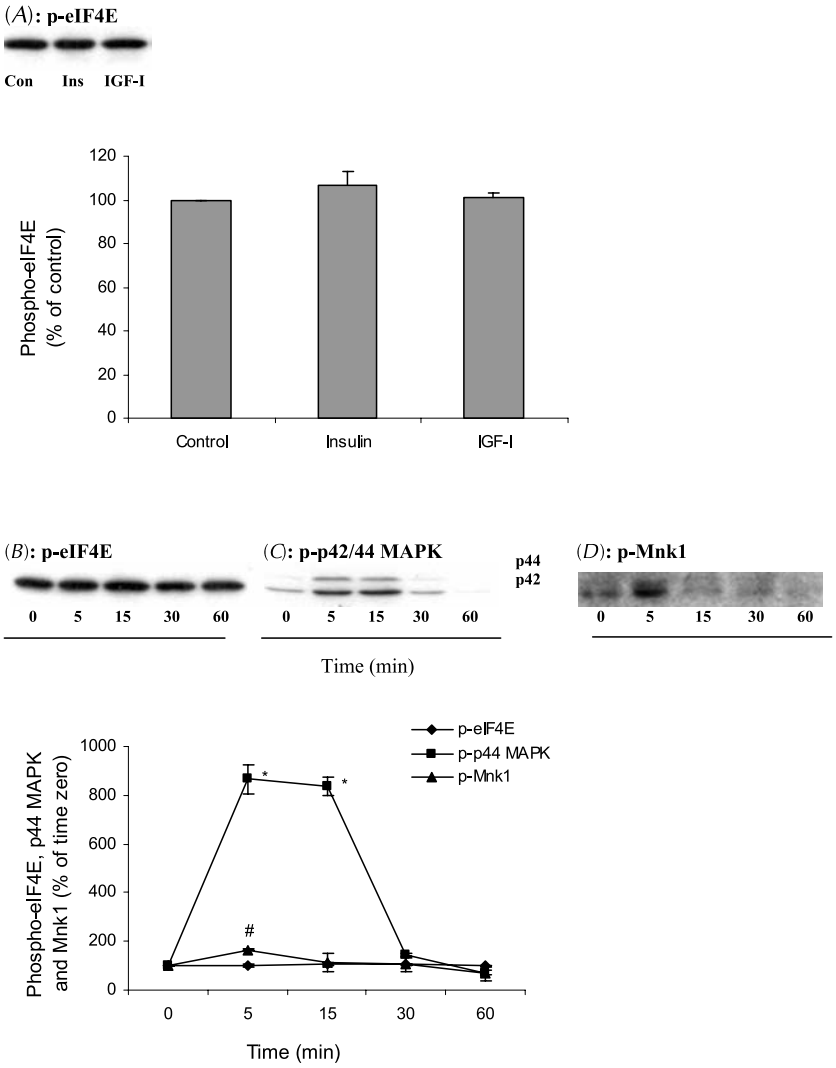


Figure 7 eIF4E phosphorylation (p-eIF4E) at Ser209 was not altered after insulin (Ins, 200 nM) or IGF-I (130 nM) treatment in nutrient-deprived C2C12 myotubes (A). In a 0–60 min time course study of insulin stimulation (200 nM), eIF4E phosphorylation was not altered (B), but p42/44 MAPK phosphorylation (p-p42/44 MAPK) and Mnk1 phosphorylation (p-Mnk1) were transiently elevated (C and D). Cells were first maintained in DPBS for 3 h on day 6 of differentiation, and then treated with insulin or IGF-I for 40 min (A) or treated with insulin for the indicated periods of time (0–60 min) (B, C and D). eIF4E phosphorylation at Ser209, p42/44 MAPK phosphorylation at Thr202/Tyr204 and Mnk1 phosphorylation at Thr197/202 in the cell lysate were analyzed by SDS-PAGE and Western blotting using phospho-specific antibodies. Con, control. * or # $P<0{\cdot}05$ relative to corresponding values of time zero.

the binding of eIF4E with 4EBP1 is regulated via the PI-3 kinase pathway, but multiple branches of this pathway may exist, and not all of these are rapamycin-sensitive. Alterations in 4EBP1 phosphorylation may account for some, but not all, of the alterations in eIF4E-4EBP1 binding.

Release of eIF4E from the eIF4E–4EBP1 complex stimulates translation initiation by allowing eIF4E to bind to eIF4G (Haghihat *et al.* 1995). In the present study, a reciprocal change in the eIF4E–4EBP1 complex and the eIF4E–eIF4G complex after insulin or IGF-I stimulation was found. However, a reciprocal change between eIF4E-4EBP1 binding and eIF4E-eIF4G binding is not consistently established in all the experiments studied. A decrease/increase in the amount of eIF4E associated with 4EBP1 did not always lead to an increase/decrease in the

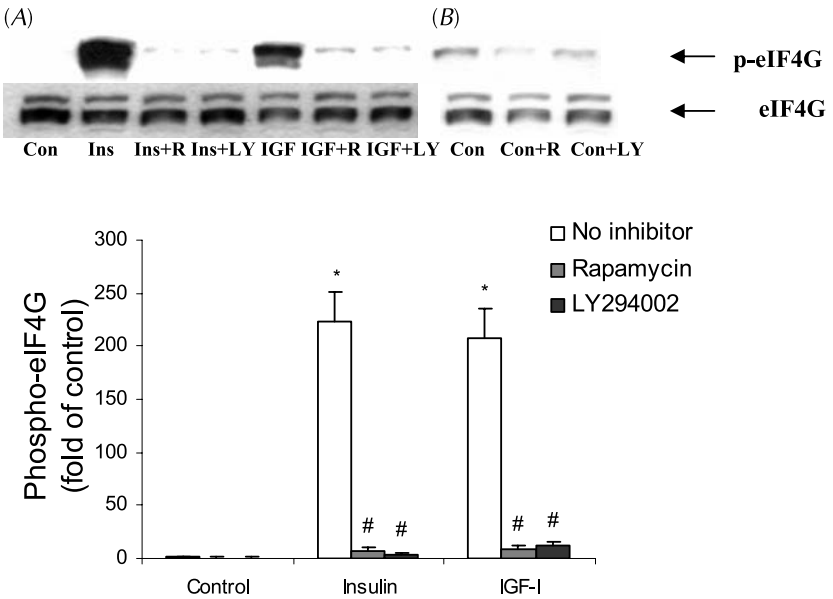


Figure 8 Insulin (Ins, 200 nM) and IGF-I (IGF, 130 nM) stimulated eIF4G phosphorylation at Ser1108 (p-eIF4G) in C2C12 myotubes in nutrient-deprived DPBS (A). The effects were attenuated and/or blocked by rapamycin (R, 128 nM) and LY294002 (LY, 32 μM). However, the basal level of eIF4G phosphorylation was not altered by the two inhibitors (B). Cells were first maintained in DPBS for 3 h on day 6 of differentiation, and then treated with insulin or IGF-I. Where indicated, rapamycin or LY294002 was added 20 min before the stimulation. Total eIF4G and eIF4G phosphorylation at Ser1108 in the cell lysate were analyzed by SDS-PAGE and Western blotting. Con, control. *P<0·05 relative to control without inhibitors; #P<0·05 relative to corresponding values without inhibitors.

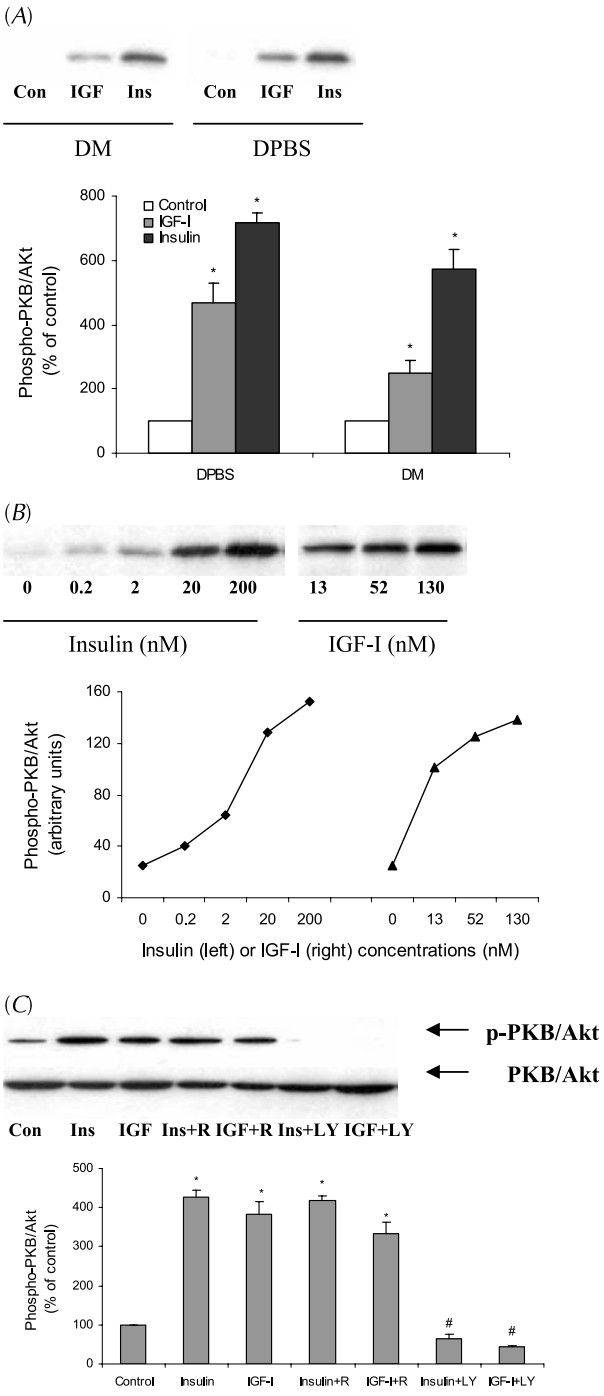
amount of eIF4E associated with eIF4G. For example, rapamycin largely prevented the insulin- and IGF-induced decrease in eIF4E-4EBP1 binding, but this did not lead to a significant decrease in eIF4E-eIF4G binding (Fig. 6). A low correlation between eIF4E-4EBP1 binding and eIF4E-eIF4G binding was also found in our previous *in vivo* study (Shen *et al.* 2002) and other investigations (Morley & McKendrick 1997, Vary *et al.* 2000), where the amount of eIF4G associated with eIF4E increased without alterations in the amount of 4EBP1 associated with eIF4E after growth factor stimulation. Clearly, under these conditions, 4EBP1 phosphorylation and a subsequent dissociation of the eIF4E–4EBP1 complex are either unnecessary, or are necessary but are not sufficient to account for the alterations of the eIF4E–eIF4G complex formation. Together with other investigations, our results suggest that dissociation of 4EBP1 from eIF4E may be regulated differently from that of association of eIF4G to eIF4E, and each of them is regulated possibly via multiple mechanisms.

Insulin and IGF-I stimulated 4EBP1 phosphorylation via the PI-3 kinase-mTOR pathway

Our results showed that insulin and IGF-I stimulated PKB/Akt phosphorylation at Ser473 in a dose-dependent manner (Fig. 9). Rapamycin, an mTOR inhibitor, blocked the insulin- and IGF-I-induced 4EBP1 phosphorylation (Fig. 4). LY294002, a PI-3 kinase inhibitor, blocked the insulin- and IGF-I-induced PKB/Akt phosphorylation at Ser473 (Fig. 9) and the insulin- and IGF-I-induced 4EBP1 phosphorylation as well (Fig. 4). Our results clearly indicate that insulin and IGF-I stimulate 4EBP1 phosphorylation via the PI-3 kinase-PKB/Akt-mTOR pathway.

Insulin and IGF-I increased mTOR phosphorylation at Ser2448 (Fig. 10). The physiological significance of the phosphorylation at Ser2448 is unclear, but the event always occurs concurrently with the phosphorylation of the mTOR downstream effector, 4EBP1, suggesting a causative correlation in the two events. However, this assumption seems to contradict the findings of other studies, where mTOR immunoprecipitates display only a modest increase or no change at all in kinase activity towards phosphorylating p70S6K at Thr389 *in vitro* following insulin stimulation (Dennis *et al.* 2001). The reason for this apparent discrepancy is unknown, but it has been suggested that mTOR kinase activity measured by *in vitro* mTOR immunoprecipitates may not represent the mTOR kinase activity *in vivo*. Regulation of mTOR kinase *in vivo* is by its associated partner proteins (Choi *et al.* 2003), and these partner proteins may be dissociated

from the mTOR functional complex during immunoprecipitation. It is also likely that the mTOR-mediated phosphorylation of p70S6K/4EBP1 may also require a signal input from other kinases. Phosphorylation of the other sites of 4EBP1/p70S6K regulated by other kinases may serve as a priming event of the mTOR-mediated phosphorylation. In addition, it is unclear whether the kinase activity is affected by antibody binding. If the mTOR kinase activity measured *in vitro* does represent the mTOR kinase activity *in vivo*, the findings of Dennis *et al.* (2001)



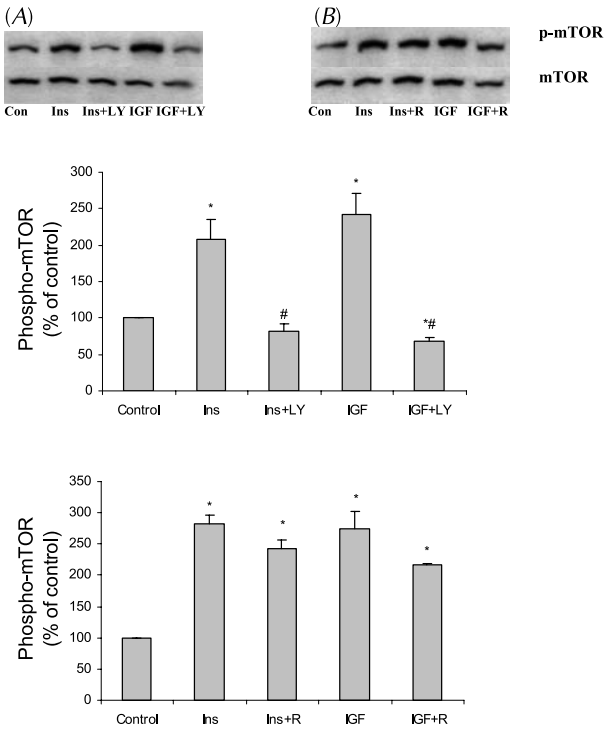


Figure 10 Insulin (Ins, 200 nM) and IGF-I (IGF, 130 nM) stimulated mTOR phosphorylation at Ser2448 (p-mTOR) in nutrient-deprived C2C12 myotubes. The stimulation was blocked by LY294002 (LY) (A), but not by rapamycin (R) (B). Cells were first maintained in DPBS for 3 h on day 6 of differentiation, and then treated with insulin or IGF-I for 40 min. Where indicated, rapamycin or LY294002 was added 20 min before the stimulation. mTOR and mTOR phosphorylated at Ser2448 were analyzed by SDS-PAGE and Western blotting using pan-mTOR antibody or phospho-mTOR antibody respectively. The data are means ± S.E.M. of three separate cell preparations, determined by densitometric scanning. The results were normalized to the amount of mTOR. *P<0·05 relative to control; #P<0·05 relative to corresponding values without inhibitors. Con, control.

Figure 9 Insulin (Ins, 200 nM) and IGF-I (IGF, 130 nM) stimulated PKB/Akt phosphorylation (p-PKB/Akt) at Ser473 in C2C12 myotubes in differentiation medium (DM) and Dulbecco's phosphate buffered saline (DBPS) (A). Insulin and IGF-I increased PKB/Akt phosphorylation at Ser473 in nutrient-deprived C2C12 myotubes (DPBS) in a dose-dependent manner (B) and this was blocked by LY294002 (LY) but not by rapamycin (R) (C). Cells were maintained in DM (A) or DPBS (A, B, and C) for 3 h on day 6 of differentiation, and then treated with insulin or IGF-I for 40 min. Where indicated, rapamycin or LY294002 was added 20 min before the stimulation. PKB/Akt phosphorylation at Ser473 in the cell lysate was analyzed by SDS-PAGE and Western blotting using a phospho-specific antibody. Con, control. *P<0·05 relative to corresponding values of control; #P<0·05 relative to corresponding values without inhibitors.

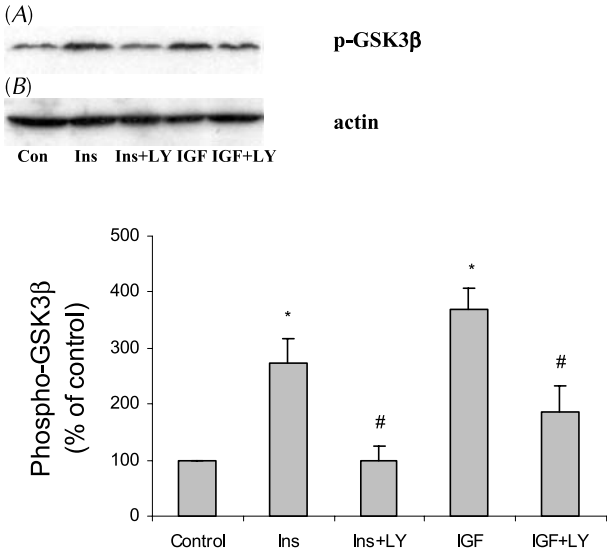


Figure 11 Insulin (Ins, 200 nM) and IGF-I (IGF, 130 nM) stimulated GSK3β phosphorylation at Ser9 (p-GSK3β) in nutrient-deprived C2C12 myotubes. The stimulation was blocked by LY294002 (LY). Cells were first maintained in DPBS for 3 h on day 6 of differentiation, and then treated with insulin or IGF-I for 40 min. Where indicated, LY294002 was added 20 min before the stimulation. GSK3β phosphorylated at Ser9 was analyzed by SDS-PAGE and Western blotting using a phospho-antibody. The data are means ± S.E.M. of three separate cell preparations, determined by densitometric scanning. The results were normalized to the amount of actin. *$P<0{\cdot}05$ relative to control; #$P<0{\cdot}05$ relative to corresponding values without the inhibitor. Con, control.

may imply that mTOR phosphorylation/mTOR kinase activity may be required but is not sufficient to account for the nutrient- and growth factor-induced 4EBP1/p70S6K phosphorylation. mTOR may function in a permissive manner and act as a priming event for other regulations. Recently, it has been suggested that the TSC1/2 complex may inhibit p70S6K activity through its upstream activator mTOR (Tee *et al.* 2002).

The insulin- and IGF-I-induced increase in the phosphorylation of mTOR was blocked by LY294002, but was unaffected by rapamycin (Fig. 10). These findings are consistent with those of Nave *et al.* (1999), where insulin stimulated the phosphorylation of mTOR at Ser2448 and activation of mTOR via the PI-3 kinase signaling pathway (Nave *et al.* 1999). Addition of rapamycin to cells in culture fails to inhibit mTOR autophosphorylation *in vivo* at Ser2481 and little or no difference in the kinase activity of mTOR is detected after rapamycin treatment (Peterson *et al.* 2000). *In vitro*, rapamycin in combination with FKBP12 does inhibit FKBP/mTOR autokinase activity, but at a much higher concentration of rapamycin than is required *in vivo* to observe this effect (Peterson *et al.* 2000).

Rapamycin blocked the insulin- and IGF-I-induced 4EBP1 phosphorylation (Fig. 4), but it did not block the insulin- and IGF-I-induced mTOR phosphorylation at Ser2448 (Fig. 10). There are two possible explanations. Either, the phosphorylation at this site is not involved in the regulation of mTOR kinase activity or rapamycin may regulate mTOR function by mechanism(s) other than those directly affecting mTOR phosphorylation (at Ser2448) and/or kinase activity. It has been suggested that the rapamycin–FKBP12 complex may displace phosphatidic acid from mTOR thereby inhibiting its activity (Mothe-Satney *et al.* 2000). Another alternative model is that the binding of the rapamycin–FKBP12 complex to mTOR triggers the activation of a phosphatase, which then acts on downstream mTOR effector molecules.

Insulin and IGF-I increased global protein synthesis in nutrient-deprived C2C12 myotubes via the PI-3 kinase pathway, but independent of the mTOR protein

Insulin (200 nM) and IGF-I (130 nM) were able to stimulate protein synthesis in C2C12 myotubes in the absence of extracellular amino acids and glucose (Fig. 2). Our findings are in agreement with those in PC12 cells, where stimulation of global protein synthesis by growth factors (epidermal growth factor (EGF) and nerve growth factor (NGF)) was observed in amino acid-starved medium (Kleijn & Proud 2000). The findings suggest that extracellular supplies of amino acids are not required for stimulation of global protein synthesis by insulin and IGF-I in C2C12 myotubes.

The insulin- and IGF-I-induced increase in protein synthesis was partially blocked by LY294002 (Fig. 2), suggesting that the stimulation was mediated via the PI-3 kinase pathway. The role of PI-3 kinase in the stimulation of protein synthesis by insulin and IGF-I has previously been demonstrated in several other studies (Dardevet *et al.* 1996, Mendez *et al.* 1996, Kitamura *et al.* 1998), but the magnitude by which PI-3 kinase inhibitors (LY294002 and wortmannin) block the stimulation varies among these studies. In rat epitrochlearis muscles, LY294002 completely abolished the insulin- and IGF-I-induced protein synthesis (Dardevet *et al.* 1996). Similarly, wortmannin, another PI-3 kinase inhibitor, has been shown to prevent the stimulation of translation by insulin in interleukin (IL)-3-starved IR/IRS-1 cells (Mendez *et al.* 1996), CHO cells and 3T3-L1 adipocytes (Kitamura *et al.* 1998).

It should be noted that, in C2C12 myotubes maintained in DPBS, insulin and IGF-I were still able to increase global protein synthesis in the presence of LY294002, suggesting that the PI-3 kinase pathway may be necessary for insulin and IGF-I to increase protein synthesis, but additional intracellular signaling pathway(s) are also involved in the insulin and IGF-I regulation of protein synthesis.

Unlike LY294002, rapamycin, an mTOR inhibitor, did not inhibit the insulin- and IGF-I-stimulated global protein synthesis in nutrient-deprived C2C12 myotubes.

Similar results were previously found in IL-3-starved IR/IRS-1 cells (Mendez *et al.* 1996). The finding that rapamycin blocked the insulin-/IGF-I-induced 4EBP1 phosphorylation without a concurrent change in global protein synthesis is unexpected, because rapamycin has been shown to inhibit the insulin-induced increase in general protein synthesis in other cell types (Dardevet *et al.* 1996). This may be due to the fact that rapamycin only blocks 4EBP1/p70S6K phosphorylation. It presumably only inhibits the translation of the mRNAs possessing the 5′TOR structure or extensive 5′cap structure (Gingras *et al.* 2001). The proportion of these mRNAs may vary among cell types. In cell types with abundant mRNA with these structures, the inhibitory effects of rapamycin on global protein synthesis are presumably more pronounced. Studies intended to distinguish the cap-dependent and cap-independent protein synthesis are necessary to elucidate this issue.

Since the insulin- and IGF-I-induced protein synthesis is affected by LY294002, but not by rapamycin, it seems that insulin- and IGF-I-induced PI-3 kinase signals may act on protein translation initiation via mechanism(s) other than activating the mTOR-4EBP1/p70S6K pathway, a well characterized mechanism *in vivo* and *in vitro*. There are at least two possible signal pathways compatible with this hypothesis. First, PKB/Akt, which is situated downstream of PI-3 kinase, but upstream of mTOR, is able to stimulate GSK3β phosphorylation, which subsequently regulates eIF2B activity and protein translation initiation (Cross *et al.* 1995). As expected, we found the PI-3 kinase inhibitor (LY294002) blocked the insulin- and IGF-I-induced PKB/Akt phosphorylation at Ser473 (Fig. 9) and blocked GSK3β phosphorylation (Fig. 11). Rapamycin, a more downstream inhibitor acting on mTOR, did not affect upstream PKB/Akt phosphorylation/activation (Fig. 9). Neither was GSK3β phosphorylation affected by rapamycin (data not shown). However, rapamycin did block the insulin- and IGF-I-induced 4EBP1 phosphorylation (Fig. 4) and p70S6K phosphorylation at Thr389/Thr421/Ser424 (Fig. 5), two known mediators of protein synthesis downstream of mTOR. Our findings suggest that the PI-3 kinase-PKB/Akt-GSK3β-eIF2B pathway may be one of the most likely mechanisms responsible for the observed insulin- and IGF-I-induced increase in global protein synthesis in nutrient-deprived C2C12 cells.

Secondly, via the PKB/Akt-Raf pathway, PI-3 kinase may cross talk with the MAPK pathway (Rommel *et al.* 1999). The involvement of the MAPK pathway in the regulation of protein synthesis has been evidenced in several studies (Kimball *et al.* 1998*b*, Kleijn & Proud 2000). The molecular mechanisms by which MAPK regulates protein synthesis remain unclear, but two possibilities have been proposed. First, MAPK can phosphorylate eIF4E through Mnk1, and eIF4E phosphorylation may regulate protein synthesis by altering its affinity to the cap structure of mRNAs, its binding to eIF4G, or other unknown mechanisms. Secondly, MAPK may be involved in the regulation of the formation of the eIF4F complex. In PC12 pheochromocytoma cells, the NGF- and EGF-enhanced binding of eIF4G to eIF4E is prevented by an inhibitor of the MAP kinase signaling pathway (Kleijn & Proud 2000). Likewise, in HEK293 cells, two structurally unrelated inhibitors of the MAP kinase pathway block eIF4G association with eIF4E caused by stimulation of cells with phorbol esters (Herbert *et al.* 2000). In the present studies, we have not investigated if the ERK1/2 MAPK is involved in the stimulation of global protein synthesis by insulin and IGF-I, but our preliminary results showed that ERK1/2 MAPK phosphorylation was transiently elevated after insulin stimulation (Fig. 7). Interestingly, eIF4E phosphorylation was not altered accordingly (Fig. 7). It remains to be investigated if the MAP kinase pathway regulates global protein synthesis by a mechanism(s) other than the regulation of Mnk1 and eIF4E phosphorylation.

In summary, we are the first group to demonstrate that insulin and IGF-I are able to regulate 4EBP1 phosphorylation, the formation of the eIF4F complex and protein synthesis in nutrient-deprived C2C12 myotubes. We also extend the previous findings in C2C12 myotubes that insulin and IGF-I regulate 4EBP1 phosphorylation via the PI-3 kinase-PKB/Akt-mTOR pathway.

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