Leucine Regulates Translation of Specific mRNAs in L6 Myoblasts through mTOR-mediated Changes in Availability of eIF4E and Phosphorylation of Ribosomal Protein S6*

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The process of translation initiation is regulated at two major steps: the binding of the 43 S preinitiation complex to the 40 S ribosomal subunit, and the binding of eIF4E to the 40 S ribosomal subunit. The mRNA binding step is mediated by a group of proteins collectively referred to as eIF4. This step is mediated by a group of proteins collectively referred to as eIF4. During this step, eIF4E binds to the m’GTP cap structure present at the 5’-end of all eukaryotic mRNAs and, through association with eIF4G, also binds to the 40 S ribosomal subunit. The mRNA binding step is regulated through changes in phosphorylation of eIF4E, with phosphorylation increasing the affinity of eIF4E for the cap structure (6) as well as by changes in the availability of eIF4E to form the active eIF4E-eIF4G complex. Changes in eIF4E availability occur through modulation of the association of eIF4E with the translational repressor, 4E-BP1. eIF4E associated with 4E-BP1 cannot bind to eIF4G and therefore does not bind to the 43 S preinitiation complex. The binding of eIF4E to 4E-BP1 is regulated through phosphorylation of 4E-BP1. A variety of hormones stimulate 4E-BP1 phosphorylation and result in dissociation of the eIF4E-eIF4G complex (reviewed in Ref. 7).

In a previous study, we showed that leucine availability in L6 myoblasts caused alterations in both the Met-tRNA$i$ and mRNA binding steps (8). In particular, leucine caused a stimulation of eIF2B activity, an impairment of eIF4E binding to 4E-BP1 and an enhancement of eIF4E binding to eIF4G. However, the changes in eIF4E availability could be dissociated from both the changes in eIF2B activity and the changes in global protein synthesis caused by leucine. Thus, global protein synthesis was regulated by alterations in eIF2B activity and were independent of changes in eIF4E availability.

The question remained as to the potential functional role of changes in eIF4E availability caused by leucine. Studies by others have suggested that changes involving eIF4E may be important in regulating the translation of mRNAs encoding specific proteins or families of proteins (reviewed in Ref. 4). eIF4E has been implicated in the regulation of translation of mRNAs containing highly structured 5’-untranslated regions, such as that coding for ornithine decarboxylase (ODC). In addition, translation of specific mRNAs is regulated through changes in phosphorylation of ribosomal protein S6 (reviewed in Ref. 9). S6 phosphorylation plays an important role in regulating the synthesis of proteins, such as elongation factors eEF1A and eEF2, which are encoded by mRNAs containing oligopyrimidine tracts at the 5’-end of the message (TOPS mRNAs). In our previous study (8), we found that leucine not
only modulated eIF4E availability, but also altered the phosphorylation state of the 70-kDa S6 protein kinase (p70S6K). Thus, leucine may regulate the translation of specific mRNAs through changes in eIF4E availability and S6 phosphorylation. The goal of the present study was to define the mechanism(s) by which leucine modulates the synthesis of the translationally regulated proteins ODC and eEF1A.

**EXPERIMENTAL PROCEDURES**

**Materials—**ECL detection reagents and horseradish peroxidase-conjugated sheep anti-mouse Ig and donkey anti-rabbit Ig were purchased from Santa Cruz Biotechnology, Inc. The antibody against p70S6k was purchased from New England BioLabs. Phosphorylated eIF4E antibody was conjugated sheep anti-mouse Ig and donkey anti-rabbit Ig were purchased from Santa Cruz Biotechnology, Inc. The antibody against Akt, and the antibody that specifically recognizes Akt phosphorylated on Ser473 were purchased from New England BioLabs. The anti- phosphoepitope antibody specific for phosphorylated S6 was a kind gift from Dr. Morris J. Birnbaum (Department of Medicine, University of Pennsylvania).

**L6 Myoblast Culture—**L6 myoblasts were grown in culture in 100-mm dishes in DMEM supplemented with 10% fetal bovine serum (HyClone Labs, Inc), 100 units/ml benzenecillin, and 100 µg/ml streptomycin sulfate. Cells were grown to approximately 70% confluence and were washed twice with phosphate-buffered saline. Serum-free DMEM lacking leucine was then added to the dishes, and the cells were returned to the incubator for 60 min. At that time the cells were randomly divided into two groups; leucine was added to the dishes in one group to a final concentration equivalent to that present in complete DMEM. When present, rapamycin at a final concentration of 90 nM was added 45 min after the change to serum-free DMEM. The cells were returned to the incubator, and 30 min later, all dishes received 10 µl of [35S]Easytag Express Protein Labeling Mix (22 cpm/µl). Thirty min after the addition of radiolabel, cells were harvested by scraping in buffer A (20 mM HEPES, pH 7.4, 100 mM KCl, 2.5% Triton X-100, 0.25% deoxycholate, 50 mM NaF, 0.2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 5 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 0.2 mM benzamidine, 0.8 µM leupeptin, and 0.6 µM pepstatin).

**Measurement of ODC Activity—**L6 myoblasts were treated as described above except they were harvested in DMEM assay buffer (50 mM Tris-HCl, pH 7.5, 2.5 mM dithiothreitol, 0.1 mM EDTA). ODC activity was determined by measuring the release of [14CO2] from [1-14C]ornithine as described previously (10).

**Measurement of eEF1A Synthesis—**L6 myoblasts were grown in culture as described above except 60-mm dishes were used and 50 µl of [35S]Easytag Express Protein Labeling Mix was added 1 h before harvest. eEF1A was immunoprecipitated by incubating 250 µl of cell homogenate with 7 µl of anti-eEF1A antibody and 118 µl of phosphate-buffered saline at 4 °C. The antibody-antigen complex was collected by incubation for 1 h with goat anti-rabbit Biomag IgG beads (PerSeptive Diagnostics). Before use, the beads were washed in 1% nonfat dry milk in buffer B (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% β-mercaptoethanol, 0.5% Triton X-100). The beads were captured using a magnetic stand and were washed twice with buffer B and once with buffer C (50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 5 mM EDTA, 0.04% β-mercaptoethanol, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). Protein bound to the beads was eluted by resuspension in SDS sample buffer and boiling for 5 min. The beads were collected by centrifugation, and the supernatants were subjected to electrophoresis on a 12.5% polyacrylamide gel. The gel was stained with Coomassie Blue dye followed by incubation in Enhance autoradiography enhancer (NER Life Science Products) for 1 h at room temperature. The gel was then rinsed with cold water, dried, and exposed to film. Films were analyzed by scanning densitometry.

**Protein Immunoblot Analysis—**Blots were developed using an Amer sham ECL Western blotting Kit and analyzed as described previously (11).

**Quantitation of 4E-BP1/eIF4E Complex—**The association of eIF4E with 4E-BP1 was quantitated by protein immunoblot analysis of eIF4E immunoprecipitates exactly as described previously (8).

**Examination of 4E-BP1 Phosphorylation in Extracts of L6 Myoblasts—**Aliquots of cell homogenate were immunoprecipitated using a monoclonal antibody raised in mice against rat 4E-BP1 as described previously (8). The immunoprecipitates were solubilized with SDS sample buffer and then subjected to protein immunoblot analysis using a rabbit anti-rat 4E-BP1 antibody.

**RESULTS**

The effect of leucine availability on global protein synthesis in L6 myoblasts was examined by incubating cells in serum-free medium lacking leucine for 1 h followed by the readdition of the deprived amino acid. Protein synthesis was then measured as the incorporation of [35S]methionine and [35S]cysteine into total cellular protein. Consistent with the results of our earlier study (8), leucine deprivation caused a reduction in protein synthesis to 52.8% of that observed in cells maintained in complete medium (Table I). Readdition of leucine rapidly returned protein synthesis to near the control value.

In addition to its effect on global protein synthesis, leucine readdition to leucine-depleted myoblasts caused a relatively greater stimulation in the activity of ODC (Fig. 1A). It has been shown previously that intracellular fluctuations in ODC activity are mediated by changes in the amount of enzyme rather than by post-translational modifications (13). In the present study, leucine readdition caused a 2.8-fold increase in ODC activity, from 0.20 ± 0.02 to 0.55 ± 0.08 nmol of [14CO2] released from [1-14C]ornithine/mg of protein/30 min, in leucine-deprived and leucine-repleted cells, respectively. In contrast, leucine stimulated global protein synthesis 1.7-fold (Table I). Thus, as assessed by enzyme activity, the increase in ODC synthesis was 1.6-fold greater in magnitude than the stimulatory effect of leucine on global protein synthesis. To confirm that the increase in ODC activity was a result of stimulated synthesis of the complete complement of amino acids, the first group were maintained in serum-free DMEM containing a complete set of amino acids, and cells in group 2 were maintained for 1 h in serum-free DMEM lacking leucine. At that time, leucine was returned to some of the cells in group 2, and 30 min later, all cells received 10 µl of [35S]Easytag Express Protein Labeling Mix (NER Research Products). Cells were harvested 30 min after the addition of Easytag Express Protein Labeling Mix, and incorporation of radiolabeled amino acids into protein was measured as described previously (43). The results are expressed as a percent of the value observed in cells maintained in serum-free DMEM containing leucine and represent the mean ± S.E. of six experiments. Within each experiment, 2–3 cultures/condition were individually analyzed.

| Condition | Protein synthesis % control |
|-----------|-----------------------------|
| Leucine-deprived | 52.8 ± 4.8* |
| Leucine readdition | 87.7 ± 5.9 |

* p < 0.05 versus leucine readdition.

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Previous studies have implicated eIF4E in the stimulation of ODC mRNA translation by the hormone insulin (15). An important mechanism for regulating eIF4E availability involves the reversible sequestration of eIF4E into an inactive complex with 4E-BP1 (16, 17). In the present study, leucine readdivision to leucine-deprived myoblasts resulted in a 70% reduction in the amount of 4E-BP1 associated with eIF4E (Fig. 1B).

Association of 4E-BP1 with eIF4E is regulated through changes in phosphorylation of 4E-BP1 with hyperphosphorylation being associated with a decrease in the binding of 4E-BP1 to eIF4E (reviewed in Ref. 18). As shown in the inset to Fig. 1C, 4E-BP1 was resolved into three bands during SDS-polyacrylamide gel electrophoresis. Previous studies have shown that the three bands represent differentially phosphorylated forms of 4E-BP1 and that the fastest migrating, or α-form, represents the least phosphorylated species of the protein, whereas the slowest migrating, or γ-form, represents the most highly phosphorylated species. Because the γ-form is the only one that does not bind to eIF4E, the data are presented as the proportion of 4E-BP1 in the γ-form. As shown in Fig. 1C, leucine readdition caused a 2.6-fold increase in 4E-BP1 phosphorylation, consistent with the observed decrease in association of 4E-BP1 with eIF4E.

Leucine readdivision to leucine-deprived myoblasts also stimulated the synthesis of eEF1A (Fig. 2A). However, whereas leucine stimulated global protein synthesis 1.7-fold, readdivision of the amino acid to leucine-deprived cells stimulated eEF1A synthesis approximately 4-fold. Thus, the increase in eEF1A synthesis relative to global protein synthesis was 2.4-fold. Synthesis of eEF1A is thought to be regulated through changes in phosphorylation of ribosomal protein S6 (19), which is phosphorylated in vivo by p70S6k (20). Activation of p70S6k by hormones occurs through phosphorylation of the protein at multiple serine and threonine residues (21). Phosphorylation results in a decrease in mobility of p70S6k during SDS-polyacrylamide gel electrophoresis, with several electrophoretic forms of the protein apparent after stimulation of cells by insulin. As described above for 4E-BP1, phosphorylation causes a decrease in the rate of migration during SDS-polyacrylamide gel electrophoresis, with the most highly phosphorylated forms exhibiting the greatest activity (21). As shown in Fig. 2B, leucine readdivision promoted a shift in p70S6k distribution, consistent with the protein becoming more phosphorylated in the presence of the amino acid. To confirm that the changes in p70S6k migration were accompanied by alterations in kinase activity, the phosphorylation state of S6 was examined using an antibody that specifically recognizes the phosphorylated form of the protein (24). Thus, mTOR plays a key role in regulating the phosphorylation state of S6 in response to changes in amino acid availability.

Recent studies have shown that a protein kinase referred to as mTOR phosphorlates 4E-BP1 both in vitro and in vivo (22, 23). Furthermore, mTOR phosphorlates p70S6k on Thr^389, a residue whose phosphorylation is associated with activation of the protein (24). Thus, mTOR plays a key role in regulating the phosphorylation of both 4E-BP1 and p70S6k. To investigate the role of mTOR in the leucine-mediated stimulation of ODC translation of 4E-BP1 was examined by protein immunoblot analysis as described under "Experimental Procedures." The results of a typical blot are shown as an inset to panel C. The positions of the α and β forms of 4E-BP1 are noted to the right. Lane 1, cells deprived of leucine; lane 2, cells deprived of leucine to which leucine was returned. The blots were subjected to densitometric analysis. Values represent the mean ± S.E. of 11 experiments/condition and reflect the total amount of 4E-BP1 (α + β) bound to eIF4E. Within each experiment, homogenates of cells from three cultures per condition were pooled before immunoprecipitation. C, cell homogenate was immunoprecipitated with a monoclonal antibody to 4E-BP1, and the phosphorylation state of 4E-BP1 was examined by protein immunoblot analysis as described under “Experimental Procedures.” The results of a typical blot are shown as an inset to panel C. The positions of the α, β, and γ forms of 4E-BP1 are noted to the right. Lane 1, cells deprived of leucine; lane 2, cells deprived of leucine to which leucine was returned. The proportion of 4E-BP1 in the γ form was assessed by densitometric analysis of the blots. The results represent the mean ± S.E. of 10 experiments/condition. Within each experiment, homogenates of cells from three cultures/condition were pooled before analysis. Hatched bars, leucine-deprived cells; solid bars, leucine-deprived cells to which leucine was returned. *p < 0.05 versus leucine-deprived cells.
activity and eEF1A synthesis, the studies shown in Figs. 1 and 2 were repeated in the presence of a specific inhibitor of mTOR, rapamycin. As shown in Fig. 3A, rapamycin did not prevent the stimulation of global protein synthesis associated with leucine readdition to leucine-deprived cells. In contrast, rapamycin prevented the leucine-induced stimulation of both ODC activity (Fig. 3B) and eEF1A synthesis (Fig. 3C). Furthermore, as shown in Fig. 3D, rapamycin prevented the leucine-induced reduction in 4E-BP1 associated with eIF4E. Finally, rapamycin not only prevented phosphorylation of p70S6k but resulted in a complete shift in p70S6k to the fastest migrating form (Fig. 3E), suggesting that p70S6k was completely dephosphorylated. The failure of leucine to stimulate p70S6k phosphorylation in the presence of rapamycin was reflected in the lack of change in S6 phosphorylation in response to leucine readdition in cells treated with the inhibitor (Fig. 3F). Overall, the results support the conclusion that leucine stimulates both 4E-BP1 and p70S6k phosphorylation through an mTOR-dependent process.

The mechanism through which leucine stimulates mTOR is unknown. Hormones such as insulin stimulate mTOR through activation of phosphatidylinositol 3-kinase (reviewed in Ref. 25). However, two recent studies (26, 27) have shown that leucine does not stimulate phosphatidylinositol 3-kinase activity in cells in culture. An alternative mechanism for the activation of mTOR by leucine may involve phosphorylation of the mTOR regulator, Akt (also known as protein kinase B). Akt is an intermediate in the insulin signaling pathway and lies downstream of phosphatidylinositol 3-kinase but upstream of mTOR (28). Activation of Akt is caused by phosphorylation of the protein at two distinct sites, one of which is Ser473. Therefore, in the present study, the effect of leucine on the phosphorylation of Akt at Ser473 was examined. As a positive control, phosphorylation of Akt in response to insulin treatment was investigated. As shown in Fig. 4, neither stimulation by insulin nor leucine had any effect on the cellular content of Akt. However, insulin, but not leucine, stimulated Akt phosphorylation. The results suggest that Akt is not a component of the signaling pathway through which leucine acts to stimulate mTOR or 4E-BP1 phosphorylation.

**DISCUSSION**

In a previous study (8), we reported that leucine availability affects both eIF2B activity and formation of the active eIF4E-eIF4G complex. Changes in global protein synthesis were shown to be directly related to changes in eIF2B activity but not to modulation of the eIF4 complex. Therefore, we proposed that leucine-induced changes in eIF4E availability might be important in regulating the translation of mRNAs coding for specific proteins. In its role as the m7GTP cap-binding protein, eIF4E is key in determining which mRNAs will be translated. Thus, those mRNAs that bind well to eIF4E presumably are translated most efficiently, and those that bind poorly are not. This fact is emphasized by studies using cells that overexpress eIF4E. In such cells, the mRNAs encoding proteins that have roles in cellular growth and development, such as ODC (15, 29), cyclin D1 (30), myc (31), and P23 (32), are preferentially translated. In contrast, decreasing the amount of eIF4E using an antisense RNA approach specifically reduces P23 expression (32). A common link among these proteins is that the mRNAs coding for them typically contain 5'-untranslated regions that are predicted to contain a high degree of secondary structure. The importance of a highly structured untranslated region in regulating the translation of these mRNAs has been demonstrated by transfecting cells with a series of vectors expressing the chloramphenicol acetyltransferase gene containing sequences in the 5'-untranslated region predicted to form different amounts of secondary structure (33). In wild type NIH-3T3 cells, increasing the secondary structure of the 5'-untranslated region caused a dramatic decrease in chloramphenicol acetyltransferase synthesis. In contrast, in NIH-3T3 cells overexpressing eIF4E, only a minor decrease in chloramphenicol acetyltransferase synthesis was observed. Thus, an increase in the amount of eIF4E relieved the translational repression caused by a structured 5'-untranslated region.

In contrast to the artificial manipulation of eIF4E availability using overexpression and antisense approaches, the present study used a more physiological approach to vary eIF4E availability, *i.e.*, through changes in association of eIF4E with 4E-BP. The release of eIF4E from the eIF4E-4E-BP complex was shown to correlate with increased ODC activity. Because ODC activity is a direct reflection of the amount of ODC protein present in the cell (13), it can be assumed that the leucine-induced increase in ODC activity represents an increase in ODC synthesis. Overall, the results suggest that leucine specifically stimulates the synthesis of ODC through an increase in eIF4E availability.

A second group of translationally regulated mRNAs is typified by ribosomal proteins and elongation factors eEF1A and eEF2. mRNAs belonging to this group usually have short 5'-untranslated regions containing little or no predicted secondary structure and have an oligopyrimidine tract at the 5' terminus. As with proteins like ODC (34), synthesis of the
ribosomal proteins is specifically increased by growth factors such as insulin (reviewed in Ref. 35). However, translational regulation of ribosomal protein synthesis is still observed in cells overexpressing eIF4E (36). In addition, in cells containing a targeted disruption of the p70S6k gene (p70S6k−/− cells), serum does not stimulate eEF1A mRNA translation (20). As expected, in p70S6k−/− cells, serum does not stimulate S6 phosphorylation even though 4E-BP1 is phosphorylated to the same extent as in cells expressing wild type p70S6k. These studies suggest that regulation of ribosomal protein synthesis occurs through an eIF4E/4E-BP1-independent mechanism that is regulated by changes in phosphorylation of ribosomal protein S6. In vivo, phosphorylation of S6 is mediated by p70S6k (20), and phosphorylation of S6 by p70S6k is associated with increased translation of TOPS mRNAs. The mechanism involved in the stimulation of TOPS mRNA translation in response to S6 phosphorylation is unknown. However, S6 present in ribosomes has been cross-linked to both initiation factors and mRNA (37), suggesting that phosphorylation of S6 might alter the interaction of the protein with either of these components and promote translation of TOPS mRNAs.

As reported previously for insulin, in the present study the leucine-stimulated phosphorylation of S6 was associated with activation of p70S6k. Furthermore, phosphorylation of S6 correlated with increased synthesis of eEF1A. eEF1A synthesis, S6 phosphorylation, and p70S6k activation were all blocked by rapamycin, suggesting that mTOR is required for this response. Indeed, mTOR has been implicated not only in the protein synthesis response to amino acids but also in the regulation of protein degradation by amino acids (38). Hara et al. (26) demonstrated that a p70S6k variant that is rapamycin-resistant is also resistant to amino acid deprivation, suggesting that amino acids signal to p70S6k through mTOR. Activation of mTOR may also be involved in the amino acid-regulated phosphorylation of 4E-BP1, because in the present study, rapamycin prevented both the phosphorylation of 4E-BP1 and the stimulation of ODC activity caused by leucine in L6 myoblasts. This idea is supported by a recent study showing that 4E-BP1 is phosphorylated both in vivo and in vitro by mTOR (22).
The question remains as to the mechanism through which amino acids activate mTOR. Hormones such as insulin activate mTOR through a phosphatidylinositol 3-kinase dependent pathway involving activation of Akt (reviewed in Ref. 39). Although the mechanism through which Akt activates mTOR is unknown, a recent study showed that stimulation of Akt causes an increase in mTOR kinase activity as well as enhances 4E-BP1 phosphorylation (28). However, in that study, the authors were unable to phosphorylate mTOR with purified Akt, suggesting that the effect of both ODC and eEF1A. The stimulation is associated with insufficient for amino acid-mediated mTOR activation. (26). Thus, it is likely that wortmannin is inhibiting a kinase downstream of mTOR, amino acid-stimulated 4E-BP1 phosphorylation was found to be both inhibited by wortmannin (42) and Chinese hamster ovary cells, amino acid-stimulated 4E-BP1 phosphorylation is inhibited by wortmannin (42) and independent of phosphatidylinositol 3-kinase and Akt activation (26). Thus, it is likely that wortmannin is inhibiting a kinase downstream of Akt or is inhibiting a step that is required but not sufficient for amino acid-mediated mTOR activation.

In summary, leucine specifically stimulates the synthesis of both ODC and eEF1A. The stimulation is associated with increased availability of eIF4E as well as phosphorylation of ribosomal protein S6. Finally, the stimulation occurs through a rapamycin-sensitive pathway, suggesting that the effect of leucine is through activation of the mTOR signaling pathway.

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