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Activation of the Mammalian Target of Rapamycin Complex 1 is Both Necessary and Sufficient to Stimulate Eukaryotic Initiation Factor 2Bε mRNA Translation and Protein Synthesis

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Abstract

In a previous study we demonstrated a requirement for activation of mTORC1 in the stimulation of eIF2Bε mRNA translation in skeletal muscle in response to resistance exercise. Although that study established the necessity of mTORC1 activation, the experimental model used did not lend itself readily to address the question of whether or not mTORC1 activation was sufficient to produce the response. Therefore, the present study was designed to address the sufficiency of mTORC1 activation, using cultures of Rat2 fibroblasts in which mTORC1 signaling was repressed by serum/leucine-depletion and stimulated by repletion of leucine and/or IGF-1. Repletion with leucine and IGF-1 caused a shift of eIF2Bε mRNA into actively translating polysomes and a stimulation of new eIF2Bε protein synthesis, but had no effect on mRNAs encoding the other four eIF2B subunits. Stimulation of eIF2Bε translation was reversed by pre-treatment with the mTORC1 inhibitor rapamycin. Exogenous overexpression of FLAG-Rheb, a proximal activator of mTORC1, also caused a re-distribution of eIF2Bε mRNA into polysomes and a stimulation of eIF2Bε protein synthesis. The stimulation of eIF2Bε mRNA translation occurred in the absence of any effect on eIF2Bε mRNA abundance. RNAi-mediated knockdown of eIF2Bε resulted in reduced cellular proliferation, a result that phenocopied the known cytostatic effect of mTORC1 repression. Overall the results demonstrate that activation of mTORC1 is both necessary and sufficient to stimulate eIF2Bε mRNA translation and that this response may represent a novel mechanism through which mTORC1 can affect mRNA translation initiation, rates of protein synthesis, and cellular growth/proliferation.

Keywords

mTOR; eIF2B; Rheb; mRNA translation

INTRODUCTION

Translation of mRNA at the ribosome and the resulting synthesis of new proteins is a fundamental process underlying cellular anabolism. Translation is divided into three distinct

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phases; initiation, elongation, and termination of which the initiation phase has been demonstrated to be rate-limiting (Mathews et al., 2000). The complex regulation of translation initiation is under the control of a family of proteins known as eukaryotic initiation factors (eIFs). One feature common to translation initiation in all eukaryotes is the delivery of the initiator methionyl tRNA (Met-tRNA$_i$) to the small 40S ribosomal subunit. Met-tRNA$_i$ forms a ternary complex with eIF2•GTP which binds to the 40S ribosomal subunit through an interaction with eIF3 to form the 43S pre-initiation complex (Hershey and Merrick, 2000). During recognition of the AUG start codon, the GTP bound to eIF2 is hydrolyzed, and the eIF2•GDP binary complex is subsequently released from the ribosome (Algire et al., 2005). In order for eIF2 to participate in successive rounds of initiation, the GDP bound to eIF2 must be exchanged for GTP, a process catalyzed by the guanine nucleotide exchange factor (GEF) eIF2B (Price and Proud, 1994, Webb and Proud, 1997). At normal cellular concentrations of magnesium, the dissociation constant for GDP is approximately 400-fold lower than for GTP (Panniers et al., 1988), therefore the exchange of eIF2-bound GDP for GTP by eIF2B activity is an absolute requirement for sustained mRNA translation and protein synthesis.

eIF2B is unique amongst cellular GEFs in both structural and regulatory complexity. The initiation factor is a heteropentameric complex composed of five unique gene products termed the α-, β-, γ-, δ-, and ε-subunits. Deletion of each of these gene products, with the exception of the α-subunit, is lethal in yeast (Hinnebusch, 1996), highlighting the importance of eIF2B activity for maintenance of cellular homeostasis. eIF2B activity is regulated via diverse mechanisms including, 1) phosphorylation of the α-subunit of (eIF2α) on Ser-51, 2) phosphorylation of eIF2Bε, 3) the redox state of pyridine dinucleotides, and 4) the abundance of eIF2B relative to its substrate eIF2 (reviewed in Webb and Proud, 1997).

Data concerning modulation of eIF2B abundance is relatively scarce; however, such a change is a potentially interesting mode of regulation, as eIF2B is found in rate-limiting amounts relative to its substrate eIF2 (Oldfield et al., 1994). Recently, it was demonstrated that eIF2Bε mRNA translation and subsequently ε-subunit protein expression are significantly elevated in rat skeletal muscle following an acute bout of resistance exercise (Kubica et al., 2005). Furthermore, the observed increase in eIF2Bε-subunit translation/protein expression was completely prevented by the bacterial macrolide rapamycin, implicating mTORC1 as a required mediator of this process. A similar increase in translation of eIF2Bδ mRNA and β-, and δ-subunit protein expression were not observed, suggesting that mTORC1-dependent translational regulation was unique to the ε-subunit. Interestingly, the minimal catalytic domain is contained within the ε-subunit (Gomez et al., 2002) and this subunit alone has been shown to have GEF activity independent of the other subunits (Fabian et al., 1997, Fabian et al., 1998, Pavitt et al., 1998, Williams et al., 2001), albeit at a reduced rate relative to the entire 5-subunit complex.

Although the previous study (Kubica et al., 2005) demonstrated that activation of mTORC1 was necessary for the stimulation of eIF2Bε mRNA translation, it did not show that mTORC1 activation was sufficient to produce the effect. Indeed, in the experimental model system used in that study, resistance exercise is known to modulate a number of cell signaling pathways (Atherton et al., 2005, Coffey et al., 2006, Kramer and Goodyear, 2007) and thus it is possible that changes in one of these in addition to mTORC1 would be necessary to stimulate eIF2Bε mRNA translation. Therefore, the purpose of the current study was to establish the sufficiency of signaling through mTORC1 to promote eIF2Bε mRNA translation. mTOR is a kinase known to integrate signals from growth factors and nutrients (Fingar and Blenis, 2004, Hay and Sonenberg, 2004) when found in the mTORC1 complex with raptor and GβL (Kim et al., 2002, Kim et al., 2003). Importantly, signaling through TORC1 has been shown to increase cell size and cell proliferation. It is well established that treatment with growth factors leads to activation of mTORC1 via the PI3K/PKB signaling pathway (Wullschleger et al., 2005).

More recently, ERK/p90RSK signaling, another pathway known to be activated by growth factors, has also been shown to promote signaling through mTORC1 (Tee et al., 2003a, Roux et al., 2004). Both PKB (Dan et al., 2002, Inoki et al., 2002, Manning et al., 2002, Potter et al., 2002) and p90RSK (Tee et al., 2003a, Roux et al., 2004) phosphorylate TSC2 and inhibit repressive GTPase activating function of the TSC1/TSC2 complex against Rheb (Castro et al., 2003, Garami et al., 2003, Inoki et al., 2003), a small GTPase that serves as a proximal activator of mTORC1 (Castro et al., 2003, Garami et al., 2003, Inoki et al., 2003, Saucedo et al., 2003, Stocker et al., 2003, Tee et al., 2003b, Tee et al., 2005). Amino acids (Hara et al., 1998, Kimball et al., 1998), in particular the branched-chain amino acid leucine (Kimball et al., 1999, Anthony et al., 2000), are also known activators of mTORC1. Leucine is believed to activate mTORC1 independently of both PI3K (Hara et al., 1998) and PKB (Kimball et al., 1999), however recent evidence suggests a role for Rheb in this process (Long et al., 2005). In the studies presented herein, the effect of leucine and/or IGF-1 treatment of Rat 2 fibroblasts on eIF2B subunit mRNA translation was examined in the presence or absence of the mTORC1 inhibitor rapamycin. Furthermore, overexpression of FLAG-Rheb was used to proximally activate mTOR to further assess the role of this kinase in eIF2Bε mRNA translation and subsequent protein synthesis. The role of eIF2Bε expression on known cellular consequences of mTORC1 signaling was also examined. It is hypothesized that amino acid-, growth factor- and Rheb-mediated activation of mTORC1 is sufficient to increase eIF2Bε mRNA translation and protein synthesis without a similar effect on mRNAs encoding other eIF2B subunits. Alteration in eIF2Bε protein expression is predicted to phenocopy cellular consequences of mTORC1 signaling.

MATERIALS AND METHODS

Reagents and Antibodies

Rapamycin was a generous gift from the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Rockville, MD). S6K1 and 4E-BP1 antibodies as well as anti-rabbit and anti-mouse horseradish peroxidase conjugated secondary antibodies were purchased from Bethyl Laboratories (Montgomery, TX). Phospho-PKB (Ser-473) polyclonal, phospho-PKB (Thr-308) (244F9) rabbit monoclonal, PKB polyclonal, phospho-ERK1/2 (Thr-202/Tyr-204) polyclonal, and ERK1/2 polyclonal antibodies were from Cell Signaling Technologies (Beverly, MA). The polyclonal antibody against Rheb (C-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Flag antibody (M2) and all other reagents were from Sigma (St. Louis, MO), except as listed below.

Cell Culture

Rat 2 fibroblasts (ATCC, Manassas, VA) were maintained in high-glucose Dulbecco's modified eagle medium (DMEM) (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Atlas Biologicals, Ft. Collins, CO) and 1% (v/v) penicillin/streptomycin (Gibco/Invitrogen). In most cases, 1.26×10^6 cells were seeded into 100-mm dishes and incubated in control culture medium overnight. The following morning cells were serum- and leucine-deprived in custom high glucose DMEM (Atlanta Biologicals, Norcross, GA) for 2 h and then treated as described in the relevant figure legends. Cell lysates were prepared for Western blotting or polysome profile analysis as described below.

For experiments involving plasmid transfection, 8.5×10^5 cells were plated in 100-mm dishes and grown in normal culture medium without penicillin/streptomycin overnight. The following day cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol with the following modifications. DNA-Lipofectamine 2000 complexes were formed using 12 μg of the appropriate vector and 30 μl of lipid reagent per

100 mm dish. Transfection was conducted for a period of 5 h at which time the medium was replaced with medium containing serum. The cells were allowed to recover overnight and then serum- and leucine-deprived for 3 h prior to processing for subsequent analysis.

For siRNA transfection experiments, 3×10^5 cells were plated in 60-mm dishes and grown in normal growth medium overnight. The following day, cells were transfected with the appropriate siRNA duplexes (30 nM) using DeliverX Plus siRNA transfection reagent (Panomics, Fremont, CA) according to the manufacturer's protocol. The scrambled control siRNA sequence was purchased from Ambion (Silencer® Negative Control #1 siRNA, Cat# AM4635). The sequence of the siRNA targeting eIF2Bε was (5'-3' sense strand) 5'-GAGGAAGAUGAAGAUGAUGtt-3'. Both scrambled control and eIF2Bε siRNA duplexes were custom synthesized by Ambion (Austin, TX).

Western Blotting

Cells were washed twice in ice-cold PBS and then scraped either directly into 1X SDS sample buffer or polysome buffer as described below. When Western blotting was done on samples collected for polysome analysis, the cells were harvested in the buffer defined below and an aliquot of the resulting lysate was centrifuged at 1 000 × g for 3 min at 4°C and a volume of the supernatant was combined with an equal volume of 2X SDS sample buffer. In either case the resulting samples were boiled for 5 min at 95°C and separated by standard SDS-PAGE. Following separation, proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane, incubated with the appropriate primary and secondary antibodies, and visualized using Pierce ECL Western Blotting Substrate (Pierce, Rockford, IL) or the ECL Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ).

RNA Isolation

Isolation of total cellular RNA was conducted using TRIzol reagent (Invitrogen, Carlsbad, CA) exactly as described in the manufacturer's protocol and resuspended in RNA storage solution (Ambion). RNA samples were analyzed for quality using the Agilent 2100 bioanalyzer microfluidics platform (Agilent Biotechnologies, Palo Alto, CA) and standard spectrophotometric techniques. The Agilent 2100 uses capillary electrophoresis to resolve RNA species and permits quantitation of individual rRNA species.

Polysome Profile Analysis

Cells were harvested in 600 μl of polysome buffer (50 mM HEPES, 250 mM KCl, 5 mM $MgCl_2$, 250 mM sucrose, 1% Triton X-100, 1.3% deoxycholate, 100 μg/ml cycloheximide, 100 U/ml SUPERase•In RNase inhibitor (Ambion)), and cell lysis was completed by agitation using an orbital rocker for 10 min at 4°C. The lysate was subjected to centrifugation at 3 000 × g for 15 min at 4°C. A portion of the resulting supernatant was combined with an equal volume of 2X SDS sample buffer and boiled at 95°C for 5 min for Western blot analysis. Yet another aliquot of the supernatant (600 μl) was layered onto a 20–47% linear sucrose gradient (50 mM HEPES, 250 mM KCl, 5 mM $MgCl_2$) and centrifuged in a Beckman SW41Ti rotor at 143 000 × g for 160 min at 4°C.

Following centrifugation the bottom of the centrifuge tube was punctured with a needle and the sucrose density gradient was displaced upward (2 ml/min) through a spectrophotometer using Fluorinert (Isco, Lincoln, NE). Optical density at 254 nm was continuously recorded (Chart Speed=150 cm/h). Two fractions representing the subpolysomal and polysomal portions of each gradient were collected directly into an equal volume of TRIzol reagent. RNA was extracted from each fraction as described in the manufacturer's protocol and resuspended in RNA Storage Solution. RNA samples were analyzed for quality as described above.

Quantitative Real-Time PCR

qRT-PCR was conducted on both the RNA sample derived from intact cells and the RNA samples isolated from the subpolysomal and polysomal fractions of sucrose density gradients. In both cases an equal quantity of RNA from each sample was converted to cDNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). The resulting cDNA was assayed to quantify the relative abundance of various mRNA species using the QuantiTect SYBR Green Real-Time PCR kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. For assessment of individual mRNA abundance from intact cells, relative expression values were normalized to relative GAPDH mRNA expression. For analysis of individual mRNA distribution in subpolysomal versus polysomal fractions, the data were expressed as the percentage of the total relative mRNA expression in each respective fraction. In both cases, a 1:2 dilution series (1:2–1:64) of one of the experimental cDNA samples was conducted to confirm linearity of the qRT-PCR assays. All experimental cDNA samples were run at a 1:16 dilution, and in all cases the resulting values fell within the linear range of the assay. The primers sets used were: eIF2Bα forward primer, 5'-GGGCTTGCGGTTTTCATTT-3'; reverse primer, 5'-TGGAGCCCCTGGTAATTCTCT-3'; eIF2Bβ forward primer, 5'-AACTGGCTTCCAGGCTGTCA-3'; reverse primer, 5'-AGCCAAGCGAGCACAGCTCCGT-3'; eIF2Bγ forward primer, 5'-GGGCTGCCTTCTCTTGTCA-3"; reverse primer, 5'-TGAACTTGCCGTGGGTAGA-3'; eIF2Bδ forward primer, 5'-TACACAACACCTCCCAATGA-3'; reverse primer, 5'-AAAGAACTTGATGGCGTTACA; eIF2Bε forward primer, 5'-TCCCCCATCTCCAAGGACC-3'; reverse primer, 5'-TCGATCAGCGCGACATTG-3'; GAPDH forward primer, 5'-GGGCTGCCTTCTCTTGTGA-3'; reverse primer, 5'-TGAACTTGCCGTGGGTAGA-3'.

Metabolic Labeling of eIF2Bε

New eIF2Bε protein synthesis was assessed by ^{35}S-metabolic labeling and subsequent eIF2Bε immunoprecipitation followed by gel electrophoresis and autoradiography. eIF2Bε synthesis was measured 1) following leucine and IGF-1 re-supplementation in the absence or presence of rapamycin and 2) under serum and leucine starved conditions in the absence and presence of exogenous Rheb overexpression. In the leucine and IGF-1 resupplementation experiments, Rat 2 fibroblasts 1×10^6 cells were plated in 100 mm dishes such that they were 50% confluent the following day. Cells were serum and leucine starved for 1.5 h, pre-treated with rapamycin or vehicle control for 30 min and then re-supplemented with leucine and IGF-1 for 60 min. During the final 30 min of the re-supplementation period the cells were treated with [^{35}S]Met/[^{35}S]Cys (200 μCi) Express Protein Labeling Mix (Perkin Elmer, Wellesley, MA). Cells were washed twice with ice-cold PBS and then lysed in 600 μl of homogenization buffer (20 mM HEPES (pH 7.4), 2 mM EGTA, 50 nM NaF, 100 mM KCl, 0.2 mM EDTA, 50 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM dithiothreitol, 0.5 mM sodium vanadate, 2.5% Trition X-100, 0.25% deoxycholate). Lysates were centrifuged at 1 000 × g at 4°C for 3 min. The resulting supernatant (500 μl) was rocked overnight at 4°C in the presence of 5 mg of an affinity-purified mouse monoclonal anti-eIF2Bε antibody generated in our laboratory. The following day, complexes were captured using goat-anti mouse Biomag beads (Qiagen) and eluted in SDS-PAGE sample buffer. Proteins were subjected to SDS-PAGE electrophoresis. The resulting gels were dried and subjected to autoradiography. In the Rheb transfection studies, Rat 2 cells (5.0×10^5 cells) were plated in 100 mm dishes such that they would be 30% confluent the following day. Cells were transfected with pRK7 empty vector or pRK7-FLAG-Rheb as described above and allowed to recover overnight. The following day, cells were serum and leucine starved for 2 h and then treated with [^{35}S]Met/Cys (200 μCi) Express Protein Labeling Mix (Perkin Elmer, Wellesley, MA) and processed as described above.

Measurement of Global Rates of Protein Synthesis

Rat 2 fibroblasts (3×10^5) were plated in 60mm dishes and incubated overnight in normal growth medium. The following day, the cells were transfected using either scrambled or eIF2Bε siRNAs at a concentration of 30 nM with a DeliverX Plus siRNA Transfection Kit (Panomics). Cells were allowed to recover for 24 hours and were then metabolically labeled for 30 min using [^{35}S]Met/Cys (100 uCi/ml). Cells were homogenized and the resulting lysate was applied to absorbent filters. Protein was precipitated in the filter with ice-cold 10% TCA followed by washing thrice with ice-cold 5% TCA. Filters were then dried, protein was solubilized, and the amount of radioactivity present in the sample was measured by scintillation counting. DPM were normalized to overall protein levels in the lysate and are reported as % of the control.

Measurement of eIF2B Activity

The guanine nucleotide exchange activity of eIF2B was measured in cell homogenates as described previously (Kimball et al., 1989)

Statistics

All data sets were assessed for potential outliers using a Grubbs test. Treatment comparisons were conducted using a one-way ANOVA (GraphPad Prism 4, San Diego, CA). If the overall ANOVA reached statistical significance at a 95% confidence level, a Tukey multiple comparison test was applied to assess significant differences ($p<0.05$) between the various treatment groups.

RESULTS

Repletion of leucine and/or IGF-1 to the culture medium stimulates signaling upstream and/or downstream of mTORC1

The well-established effects of the branched-chain amino acid leucine (Hara et al., 1998, Kimball et al., 1999) and the growth factor IGF-1 (Rommel et al., 2001) on mTORC1 signaling were confirmed in the Rat 2 fibroblast cell line. Cells were serum-and leucine-starved for 2 h and then leucine (1 mM), IGF-1 (2.67 nM) or the combination of both leucine and IGF-1 were added back to the medium for a period of 60 min. Control cells remained in medium lacking serum and leucine and thus were starved for a total of 3 h. Treatment with leucine, IGF-1, or the combination of leucine and IGF-1 increased the phosphorylation status of the mTORC1 targets S6K1 and 4E-BP1 (Figure 1A). For both S6K1 and 4E-BP1, administration of IGF-1 alone or the combination of IGF-1 and leucine caused the greatest extent of hyperphosphorylation while leucine alone had an intermediate effect. As expected, repletion with leucine alone did not promote PKB or ERK1/2 phosphorylation. In contrast, treatment with IGF-1 alone or the combination of IGF-1 and leucine for 60 min promoted phosphorylation of PKB on Ser-473; however, no change in ERK1/2 (Thr-202/Tyr-204) phosphorylation was observed under these conditions (Figure 1A). Because it has been demonstrated that IGF-1 activation of ERK1/2 is transient (Porras et al., 1998, Duan, 2003, Lassarre and Ricort, 2003, Ahmad et al., 2004, Zheng and Quirion, 2004, Subramaniam et al., 2005) a detailed time course analysis was conducted to clearly establish the temporal relationship between IGF-1 treatment and the phosphorylation of PKB, ERK1/2, S6K1 and 4E-BP1 (Figure 1B). PKB (Ser-308) phosphorylation was maximally stimulated 5–10 min following IGF-1 treatment and remained elevated over control throughout the 30 min time course. An increase in PKB phosphorylation on the Ser-473 residue was evident by 5 min, maximal at 10–15 min and remained elevated 30 min following stimulation. ERK 1/2 phosphorylation on Thr-202 and Tyr-204 residues was transiently elevated 10 min and 15 min following treatment with IGF-1 alone, or leucine plus IGF-1, but returned to control values by

30 min after treatment. In all of the treatment groups, increased phosphorylation of S6K1 and 4E-BP1 occurred as early as 10 min following treatment and remained elevated for the remainder of the time course analyzed. As before, treatment with IGF-1 alone or the combination of leucine and IGF-1 elicited the maximal response, while administration of leucine alone had an intermediate effect. Thus, Rat 2 fibroblasts are a model system in which leucine and IGF-1 stimulate signaling through mTORC1, likely through cellular signaling pathways that converge at, or possibly lie upstream of, Rheb.

Repletion of leucine and/or IGF-1 in the culture medium increases translation of eIF2Bε mRNA, but has no effect on mRNAs encoding other eIF2B subunits

In order to assess the effect of leucine and/or IGF-1 on translation of mRNAs encoding the five subunits of eIF2B, sucrose density gradients were used to separate mRNAs being actively translated (i.e. present in polysomes) from untranslated mRNA (i.e. not associated with polysomes). Tracings obtained during fractionation of the gradients suggested that replacement of leucine and/or IGF-1 stimulated initiation of mRNA translation, as repletion caused a general reduction in the height of peaks corresponding to the 40S and 60S ribosomal subunits and 80S monomers compared to the serum- and leucine-starved control fibroblasts (Figure 2A). Consistent with the extent of phosphorylation of the mTORC1 targets S6K1 and 4E-BP1, leucine caused a more modest decrease in the height of the individual subpolysomal peaks compared to the IGF-1 and leucine plus IGF-1 treatment groups. The population of 80S monomers appeared to be particularly sensitive to repletion of leucine and/or IGF-1.

Two samples were collected directly into TRIzol from each sucrose density gradient fraction corresponding to the subpolysomal and the polysomal fractions, respectively. RNA was obtained using standard extraction procedures and shown to be of high quality using an Agilent 2100 Bioanalyzer (data not shown). qRT-PCR was then used to assess the percentage of the relative mRNA abundance in the polysomal fractions for each of the five mRNAs encoding eIF2B subunits (Figure 2B). Under serum- and leucine-depletion conditions, approximately 50% of the total eIF2Bε mRNA was present in the polysomal fraction in control cells. Repletion of leucine (70% in polysomes; $p<0.05$ *vs.* control), IGF-1 (73% in polysomes; $p<0.05$ *vs.* control) or the combination of leucine and IGF-1 (69% in polysomes; $p<0.05$ *vs.* control) led to a significant re-distribution of the ε-subunit mRNA out of the subpolysomal fraction and into the actively translating polysomal fraction. Regulation of eIF2Bε mRNA polysome distribution was unique among the mRNAs encoding eIF2B subunits, as 70–80% of the mRNA encoding the other four subunits of the protein was present in the polysomal fraction, regardless of the treatment condition.

To ensure that the shift in eIF2Bε mRNA into actively translating polysomes was not a result of an increase in the total abundance of the message (e.g. transcriptional regulation), the study was repeated and total RNA was isolated directly from intact cells. RNA quality was assessed using an Agilent 2100 Bioanalyzer (data not shown) and SYBR Green qRT-PCR was used to assess relative eIF2Bε mRNA expression as normalized to GAPDH. Repletion of leucine and/or IGF-1 had no effect on the relative abundance of eIF2Bε-subunit mRNA expression (Figure 2C). Similarly, no change in the relative abundance of eIF2Bδ mRNA was observed (Figure 2C). These results suggest the observed increase in polysome association of eIF2Bε mRNA represented enhanced translation of existing mRNA.

The mTORC1 inhibitor rapamycin completely prevents the leucine and IGF-1-induced re-distribution of eIF2Bε mRNA into polysomes and increase in eIF2Bε protein synthesis

To provide evidence that mTORC1 signaling is required to mediate the shift of eIF2Bε-subunit mRNA into actively translating polysomes, a subset of cells were pre-treated with 100 nM rapamycin 30 min prior to repletion of leucine and IGF-1. As demonstrated above, repletion

of leucine and IGF-1 caused an increase in phosphorylation of S6K1 and 4E-BP1 (Figure 3A); however, pre-treatment with rapamycin prior to the addition of leucine and IGF-1 caused a dramatic reduction in phosphorylation of S6K1 and 4E-BP1. Tracings from the polysome profile analysis (Figure 3B) confirmed the positive effect of leucine and IGF-1 on translation initiation as assessed by a decrease in the height of peaks corresponding to the 40S and 60S ribosomal subunits and 80S monomers. Pre-treatment with rapamycin partially prevented the reduction in the height of the subpolysomal peaks associated with leucine and IGF-1 repletion. Once again, RNA was isolated from the subpolysomal and polysomal fractions of each sample and quality control analysis was conducted (data not shown). SYBR Green qRT-PCR showed that relative to the serum- and leucine-deprived controls (57% in polysomes) repletion of leucine and IGF-1 caused a significant increase in the polysomal distribution of eIF2Bε mRNA (85% in polysomes; $p<0.01$ *vs.* control), a shift that was completely prevented by pre-treatment with rapamycin (62% in polysomes; $p<0.01$ *vs.* leucine and IGF-1) (Figure 3C). In agreement with the mRNA polysome association data, metabolic labeling of newly produced proteins with [^{35}S]Met/Cys followed by eIF2Bε immunoprecipitation, polyacrylamide gel electrophoresis (PAGE) and autoradiography revealed that eIF2Bε protein synthesis was significantly increased (32%, $p<0.05$) by repletion with leucine and IGF-1 (Figure 3D). This increase in nascent eIF2Bε protein production was completely prevented by pre-treatment with rapamycin ($p<0.05$).

Overexpression of exogenous FLAG-Rheb stimulates re-distribution of eIF2Bε mRNA into actively translating polysomes and an increase in new eIF2Bε protein synthesis

Transfection of Rat 2 fibroblasts with pmaxGFP along with counterstaining for F-actin with Alexa Fluor 350 phallodin revealed >99% transfection efficiency in this cell line (data not show). The cells were then transfected with pRK7-FLAG-Rheb. Overexpression of exogenous FLAG-Rheb was confirmed by Western blotting and was shown to stimulate hyperphosphorylation of the mTORC1 targets S6K1 and 4E-BP1 in serum-starved cells (Figure 4A). SYBR Green qRT-PCR showed that relative to cells transfected with the pRK7 empty vector control plasmid (44% in polysomes) Rat 2 fibroblasts overexpressing FLAG-Rheb exhibited a significant increase in eIF2Bε mRNA polysome association (60% in polysomes; $p<0.001$ *vs.* control) while displaying no change in the polysomal distribution of GAPDH mRNA (Figure 4B). Similar to the response following leucine and/or IGF-1 repletion, overexpression of FLAG-Rheb had no effect on the total abundance of eIF2Bε-subunit mRNA from intact cells when expression was normalized to GAPDH (Figure 4C) suggesting that transcriptional control did not contribute to increased polysome association. In addition, metabolic labeling with [^{35}S]Met/Cys demonstrated that FLAG-Rheb overexpression caused an increase (186% of control) in new eIF2Bε protein synthesis compared to cells transfected with the empty vector control (Figure 4D). To confirm that the effect of Rheb overexpression on eIF2Bε expression was due to changes in mTORC1 signaling, rapamycin was added to the culture medium of cells transfected with either the pRK7 empty vector control plasmid or the FLAG-Rheb plasmid prior to harvest. As shown in Figure 4E, treatment with rapamycin had no effect on Rheb expression, but dramatically attenuated the Rheb-induced increase in S6K1 phosphorylation. Moreover, rapamycin blocked completely the Rheb-induced shift of eIF2Bε mRNA into the polysome fraction, demonstrating the Rheb was acting through mTORC1 to upregulate eIF2Bε mRNA translation (Figure 4F).

RNAi knockdown of eIF2Bε leads to reduction in Rat 2 fibroblast proliferation

Previous work has demonstrated that repression of mTORC1 signaling leads to reduced cellular proliferation (e.g. Fingar et al., 2004). mTORC1 signaling is highly elevated in Rat 2 fibroblasts grown in normal growth medium as assessed by hyperphosphorylation status of S6K1 and 4E-BP1 (data not shown), suggesting a relatively high rate of cellular proliferation, increased eIF2Bε mRNA association with actively translating polysomes and elevated eIF2Bε protein

expression. In order to establish a functional relationship between mTORC1-mediated elevation in cellular proliferation and mTORC1-mediated increases in eIF2Bε expression, eIF2Bε was targeted by RNAi and cell number was monitored by haemocytometer counts over time. Compared to cells transfected with a scrambled control siRNA, Rat 2 fibroblasts treated with a siRNA duplex targeting eIF2Bε led to a significant reduction in eIF2Bε expression as assessed by Western blot (Figure 5A). RNAi-mediated knockdown of eIF2Bε resulted in a decrease in eIF2Bε activity (Figure 5B) and global rates of protein synthesis (Figure 5C). Cells transfected with eIF2Bε siRNA also exhibited significantly reduced cellular proliferation rates (Figure 5D), an effect that was apparent within 48 h post-transfection (66% of control, $p<0.001$) and maximal at 72 h post-transfection (57% of control, $p<0.001$).

DISCUSSION

The major finding of the current study is that signaling through mTORC1 is both necessary and sufficient to stimulate eIF2Bε mRNA translation and eIF2Bε protein synthesis in Rat 2 fibroblasts. The observed shift of eIF2Bε message into actively translating polysome complexes appears to be indicative of translational control as there was no observed change in total eIF2Bε-subunit mRNA expression that would suggest a role for transcriptional control and subsequent bulk movement of this specific mRNA into polysomes. The ability of the mTORC1 inhibitor rapamycin to completely prevent the stimulatory effect of leucine and IGF-1 on eIF2Bε-subunit mRNA translation strongly suggests the necessity of mTOR-mediated signaling in this process. Furthermore, leucine and IGF-1 repletion stimulates an increase in eIF2Bε protein synthesis as assessed by ^{35}S metabolic labeling. The sufficiency of mTORC1 to mediate the effect is established by the ability of exogenous overexpression of a FLAG-tagged version of the small GTPase Rheb to stimulate eIF2Bε mRNA translation and protein synthesis.

These results support earlier published work in rat gastrocnemius muscle following acute resistance exercise (Kubica et al., 2005), a physiological perturbation also known to activate mTORC1 signaling (Hernandez et al., 2000, Nader and Esser, 2001, Bolster et al., 2003, Parkington et al., 2003). In the aforementioned study, eIF2Bε mRNA translation and protein expression were increased following exercise in a rapamycin-sensitive manner. No change in the translation of eIF2Bδ mRNA or protein expression of the β- or δ-subunits of eIF2B were observed, suggesting that mTORC1-dependent translational regulation is unique to the catalytic eIF2Bε subunit. The results presented herein extend those observations in that activation of mTORC1 by leucine and/or IGF-1 had no effect on polysome association of mRNAs encoding the α-, β-, γ-, or δ-subunits of eIF2B. Interestingly, under control conditions the mRNAs encoding the α- (77% in polysomes), β- (83% in polysomes), γ- (88% in polysomes) and δ-subunits (80% in polysomes) were considerably more abundant in the polysomal fraction compared to eIF2Bε mRNA (55% in polysomes). Nearly identical results were observed when comparing the polysomal association of the δ- and ε-subunit mRNAs in rat skeletal muscle in sedentary rats. Thus, translation of the mRNA encoding eIF2Bε is specifically affected by changes in signaling through mTORC1.

The results presented herein provide evidence for the sufficiency of mTORC1 in mediating translational control of eIF2Bε expression and the upstream regulators that can participate in this regulation. The ability of four unique stimulators of mTORC1, namely resistance exercise, leucine, IGF-1, and Rheb to cause re-distribution of eIF2Bε mRNA into polysomes strongly suggests that signaling through mTORC1 is sufficient to induce translational control of this message. It is known that growth factors (IGF-1) and amino acids (leucine) activate mTORC1 through independent signaling pathways. As expected, IGF-1 treatment resulted in increased phosphorylation of S6K1 and 4E-BP1, two well-defined downstream targets of mTORC1 and induced phosphorylation of PKB and ERK1/2, two upstream activators of mTORC1. In

contrast, leucine promoted signaling through mTORC1 in a PKB- and ERK1/2-independent manner. In comparison to the maximal effect on mTORC1 signaling observed upon treatment with IGF-1 or the combination of leucine and IGF-1, stimulation with leucine alone caused an intermediate effect on S6K1 and 4E-BP1 phosphorylation status. Despite the observed differences in the mechanism and extent of mTORC1 activation, treatment with leucine alone, IGF-1 alone, or the combination of leucine and IGF-1, promoted a statistically identical effect on association of eIF2Bε mRNA with actively translating polysomes. The common proximal activator of mTORC1 downstream of both growth factor and amino acid stimulation is the small GTPase Rheb. Overexpression of FLAG-Rheb was sufficient to cause a re-distribution of eIF2Bε mRNA and increased eIF2Bε protein synthesis under serum- and leucine-free conditions. Taken together, the results support the hypothesis that a modest activation of mTORC1 via at least two independent signaling pathways converging on Rheb is sufficient to induce increased eIF2Bε translation and subsequent protein synthesis. It is important to note that the current study does not discriminate between localization of eIF2Bε mRNA between lighter polysome aggregates and heavier polysome populations, thus the contribution of particular upstream activators or the extent of mTORC1 activation could affect the rate of eIF2Bε-subunit translation in this manner.

In the present study, RNAi knockdown of eIF2Bε had a moderate cytostatic effect on Rat 2 fibroblasts, a cellular consequence that phenocopies the previously published effect of mTORC1 repression on cellular proliferation. However, although this result is consistent with the idea that mTORC1 regulates proliferation in part by controlling eIF2Bε expression, the results at present simply provide a correlation among the observations. Further studies will be required to establish a direct link between mTORC1-mediated changes in eIF2Bε expression and cell proliferation.

In conclusion, repletion of leucine and/or IGF-1 to deprived cells causes an increase in eIF2Bε mRNA translation and eIF2Bε protein synthesis in Rat 2 fibroblasts in a rapamycin-dependent manner. Furthermore, overexpression of the small GTPase Rheb, the most proximal upstream activator of mTORC1, is sufficient to confer this increase in eIF2Bε message re-distribution into polysomes and new eIF2Bε protein production. These results strongly suggest that signaling through mTORC1 is both necessary and sufficient to drive translational control of the catalytic subunit of eIF2B. A similar result has been previously observed in rat gastrocnemius muscle following a physiological model of resistance exercise, suggesting a novel mechanism by which mTORC1 signaling may regulate translation initiation, global rates of protein synthesis and cellular growth. Several critical questions remain including the identity of the mTORC1 effectors involved in eIF2Bε translational control and the elements within the ε-subunit message that confer this mode of regulation.

ABBREVIATIONS

eIF, eukaryotic initiation factor
Met-tRNA$_i$, initiator methionyl-tRNA
GEF, guanine nucleotide exchange factor
mTOR, mammalian target of rapamycin
mTORC1, mTOR complex 1
Raptor, regulatory associated protein of mTOR
GβL, G protein β-subunit-like protein
PI3-K, phosphoinositide 3-kinase
PKB, protein kinase B (also known as Akt)
ERK, extracellular signal-regulated kinase
p90RSK, p90 ribosomal protein S6 kinase
TSC, tuberous sclerosis complex 1

Rheb, Ras homologue enriched in brain
S6K1, ribosomal protein S6 kinase 1
4E-BP1, eukaryotic initiation factor 4E-binding protein 1

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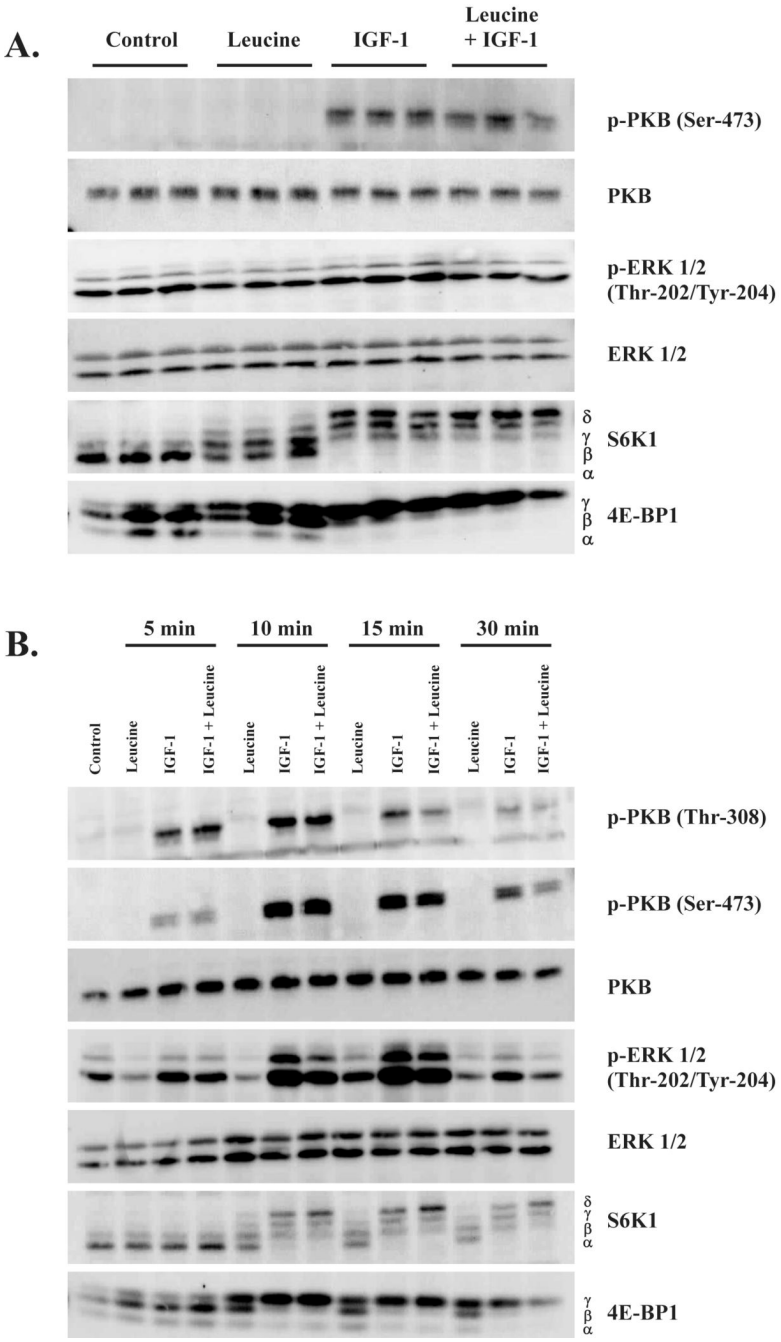

Fig 1.

Effect of serum- and leucine-depletion or leucine and/or IGF-1 re-supplementation signaling upstream and downstream of mTOR. (A) Phosphorylation of both upstream activators and downstream effectors of mTORC1 is evident 60 min following treatment with the known mTORC1 stimulators leucine and/or IGF-1. Cells were either serum- and leucine-depleted for 3 h or serum and leucine depleted for 2 h and then re-supplemented with leucine (1mM), IGF-1 (2.67 nM) or the combination of both leucine and IGF-1 for a period of 60 min. Representative blots for PKB phosphorylated on Ser-473 (p-PKB (Ser-473)), PKB total, ERK1/2 phosphorylated on Thr-202/Tyr204 (p-ERK1/2 (Thr-202/Tyr-204)), ERK1/2 total, S6K1, and 4E-BP1 are shown. In all cases the results are representative of at least three separate

experiments. (B) Rat 2 fibroblasts were treated as described in Panel A for the indicated period time. Representative blots for PKB phosphorylated on Thr-308 (p-PKB (Thr-308)), PKB phosphorylated on Ser-473 (p-PKB (Ser-473)), PKB total, ERK1/2 phosphorylated on Thr-202/Tyr204 (p-ERK1/2 (Thr-202/Tyr-204)), ERK1/2 total, S6K1, and 4E-BP1 are shown. In all cases the results are representative of at least three experiments.

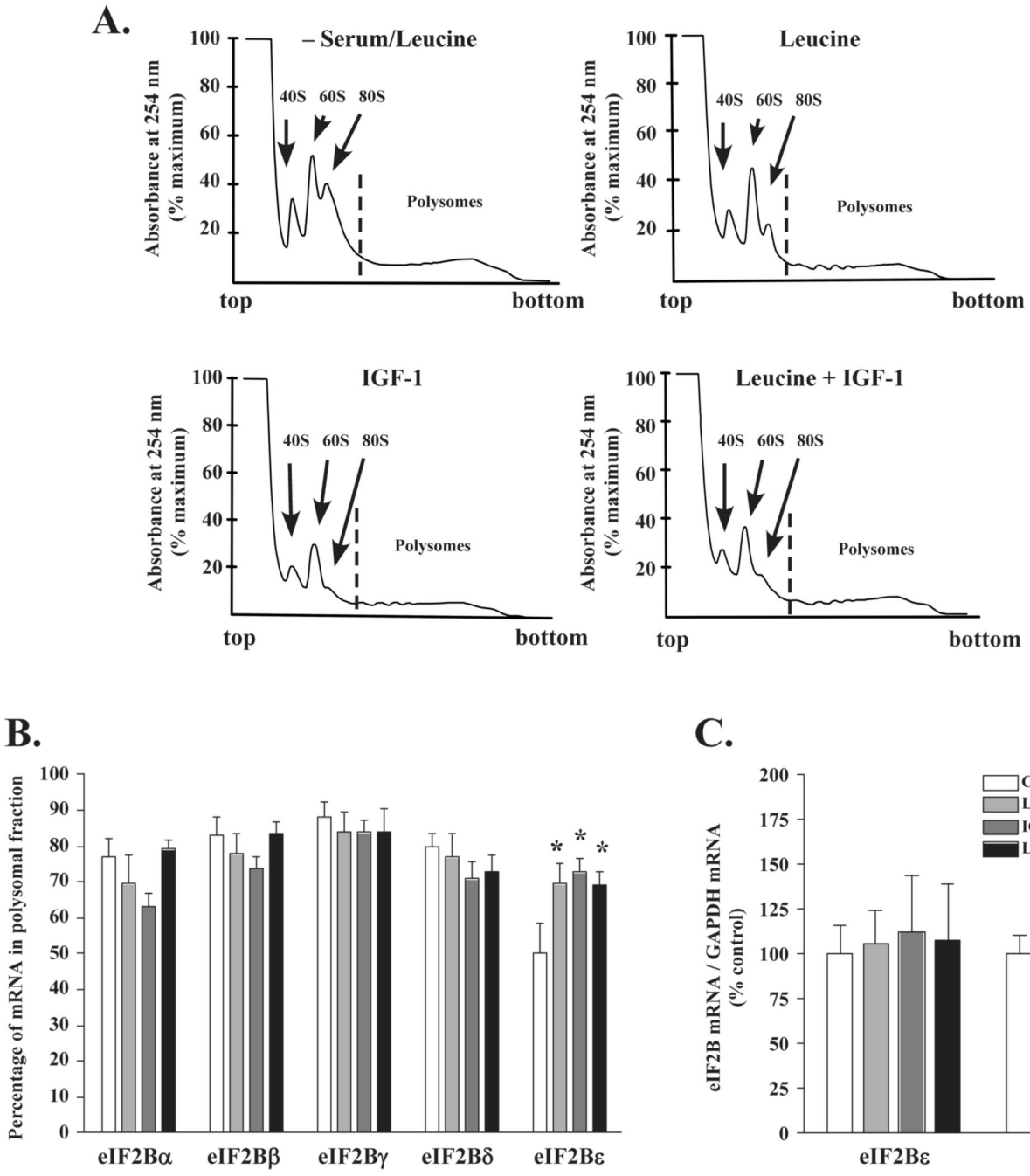

Fig 2.

Repletion of leucine and/or IGF-1 to the culture medium of Rat 2 fibroblasts causes an increased association of eIF2Bε mRNA with actively translating polysome aggregates. Cells were either serum- and leucine-depleted for 3 h (Control) or serum- and leucine-depleted for 2 h and then re-supplemented with leucine (1 mM), IGF-1 (2.67 nM), or the combination of both leucine and IGF-1 for a period of 60 min. (A) Cell supernatants were analyzed by sucrose density gradient centrifugation as described under “Materials and Methods”. In all cases, the results are representative of at least 4 independent experiments. The peaks corresponding to the free (i.e. non-polysome associated) 40S subunits, free 60S subunits, 80S monosomes, and the progressively larger polysome complexes are indicated. Two fractions corresponding to the

subpolysomal portion of the gradient (left of the dashed line) and the polysomal portion of the gradient (right of the dashed line) were collected directly into TRIzol for subsequent RNA extraction. (B) RNA from the subpolysomal and polysomal fractions for each treatment group were individually reverse-transcribed to cDNA and analyzed by SYBR Green qRT-PCR using primers specific for the α-, β-, γ-, δ- and ε-subunits of eIF2B as described under "Materials and Methods". Values are expressed as the percent present in the polysomal fraction. The graphical data represents mean values ± SEM (n=4–7 per group). * $p<0.05$ *vs.* control. (C) RNA was extracted from cells scraped directly in TRIzol. RNA was reverse-transcribed to cDNA and analyzed by SYBR Green qRT-PCR using primers specific to eIF2Bεor eIF2Bε mRNA. Relative values for eIF2Bε and eIF2Bδ mRNA were normalized to GAPDH mRNA, expressed as a percentage of control and shown as mean values ± SEM (n=9 per group; n.s.).

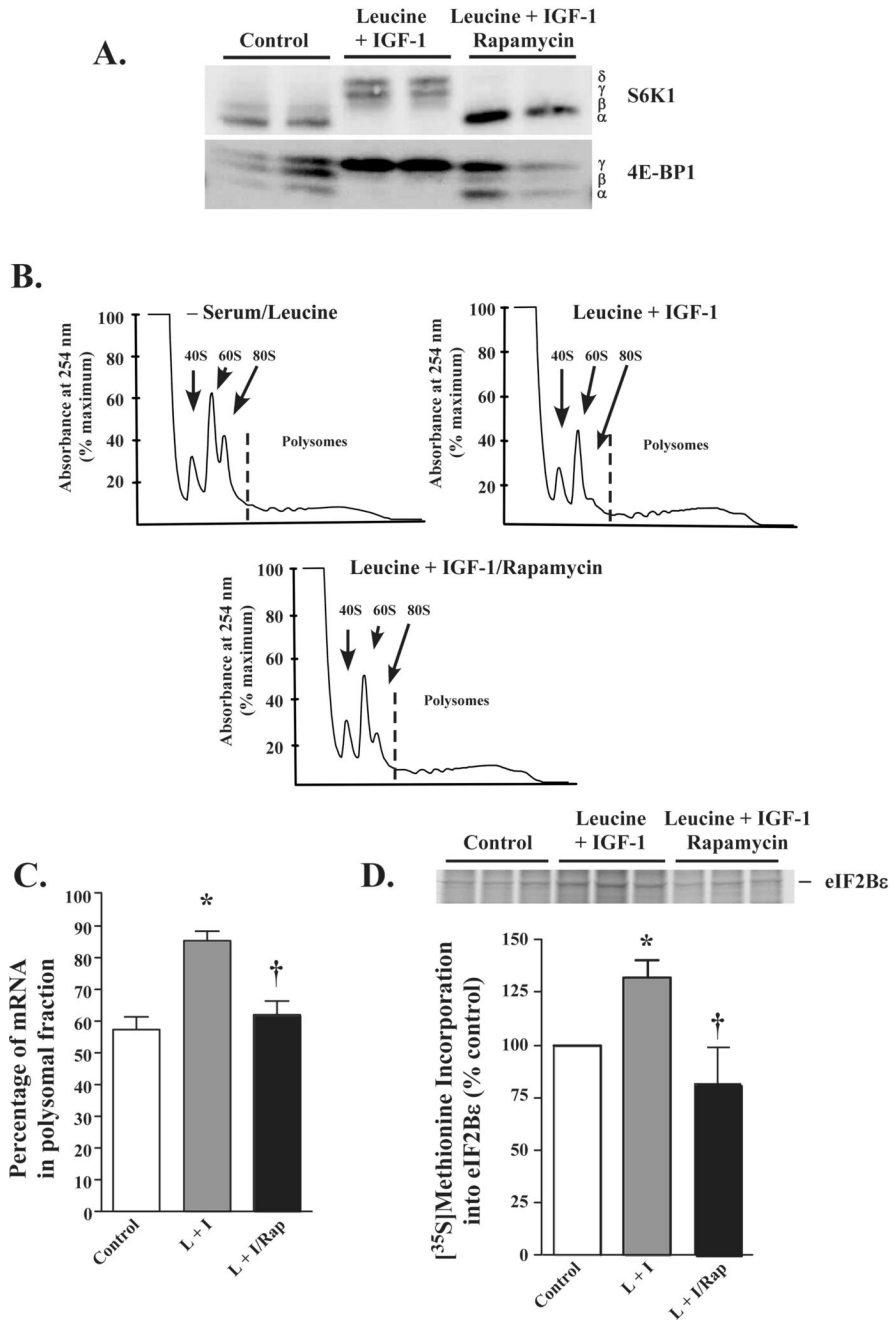

Fig 3.

Pre-treatment of Rat 2 fibroblasts with rapamycin prior to repletion of leucine and IGF-1 represses signaling through mTORC1, the shift of eIF2Bε into actively translating polysomes, and increased incorporation of [^{35}S]Met into eIF2Bε. Cells were treated as described in Fig. 1, Panel A. A subset of cells were pre-treated with the mTORC1 inhibitor rapamycin (100 nM) 30 min prior to re-supplementation with leucine and IGF-1. (A) Representative Western blot analysis of S6K1 and 4E-BP1 phosphorylation status. (B) Representative polysome profile tracings for each treatment group. The peaks corresponding to the free 40S subunits, free 60S subunits, 80S monosomes, and the progressively larger polysome complexes are indicated. Two fractions corresponding to the subpolysomal portion of the gradient (left of the dashed

line) and the polysomal portion of the gradient (right of the dashed line) were collected directly into TRIzol for subsequent RNA extraction. (C) RNA was extracted from the subpolysomal and polysomal fractions from each treatment group. The RNA from each fraction was individually reverse-transcribed to cDNA and analyzed by qRT-PCR using primers specific for the ε-subunit of eIF2B. Values are expressed as the percent present in the polysomal fraction. The graphical data represents mean values ± SEM (n=6 per group). * $p<0.001$ *vs.* control; † $p<0.01$ *vs.* L+I. (D) Incorporation of [^{35}S]Met/Cys into eIF2Bε was measured in eIF2Bε immunoprecipitates as described under “Materials and Methods”. An autoradiograph from a typical experiment is shown as an inset. Within each experiment, three separate dishes of cells was analyzed per condition, and three such experiments were performed. * $p<0.05$ *vs.* control; † $p<0.05$ *vs.* L+I..

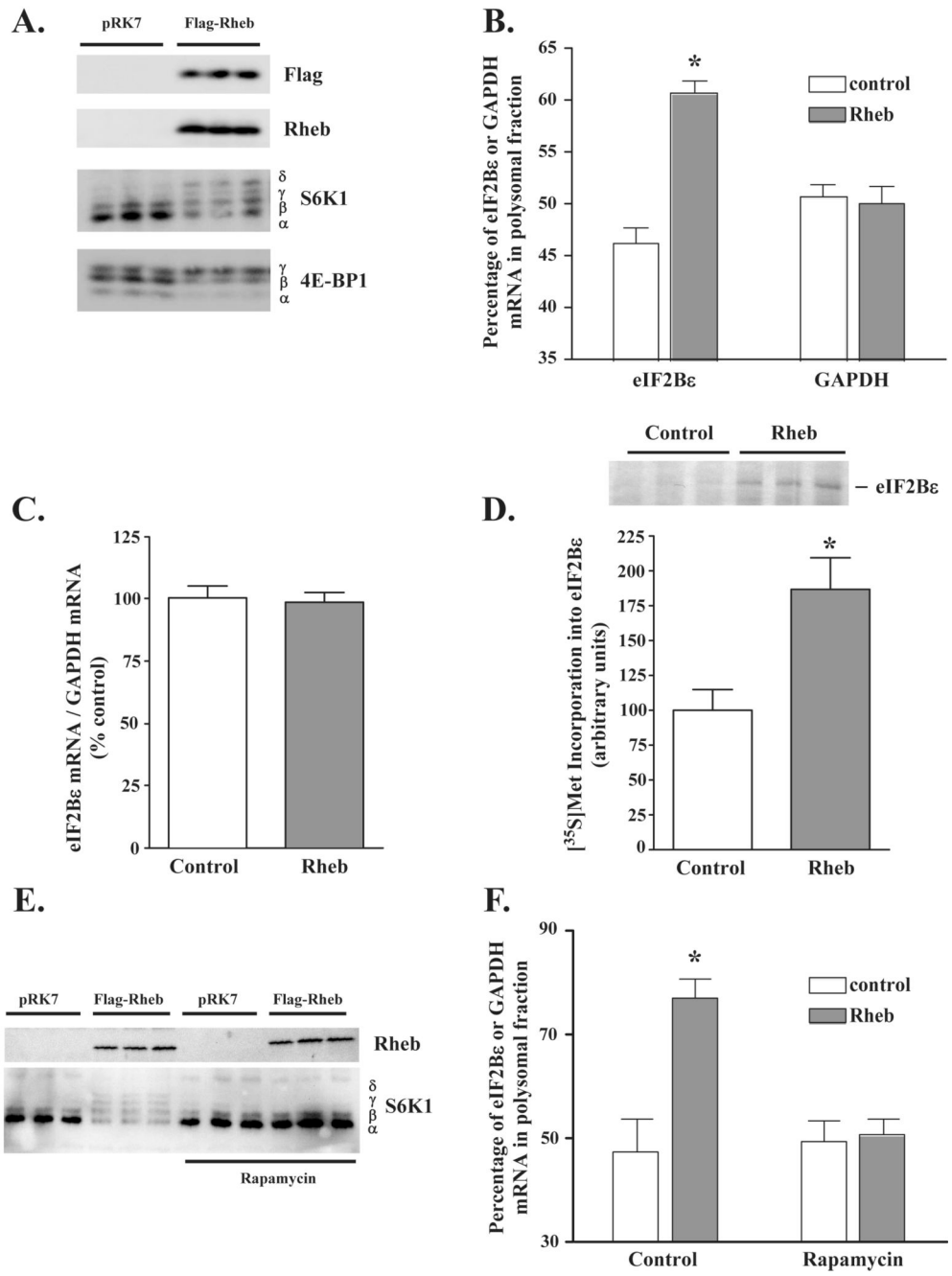

Fig 4.

Overexpression of FLAG-Rheb activates mTORC1 signaling and is sufficient to increase eIF2Bε mRNA polysome association. Rat 2 fibroblasts were transfected with Lipofectamine 2000 for 5 h and then allowed to recover overnight. Cells were then serum-starved for 3 h prior to analysis. (A) Western blot analysis of exogenously expressed (Flag) and endogenous Rheb, S6K1 and 4E-BP1. Blots representative of 3 independent experiments are shown. (B) RNA was extracted from both the subpolysomal and polysomal fractions, reversed transcribed, and analyzed for relative eIF2Bε and GAPDH mRNA abundance respectively using qRT-PCR. Values are expressed as the percent present in the polysomal fraction. The graphical data represents mean values ± SEM (n=6 per group). * $p<0.001$ *vs.* control. (C) Total RNA was

reverse-transcribed and analyzed by SYBR Green qRT-PCR using primers specific for eIF2Bε. Relative values for eIF2Bε were normalized to GAPDH, expressed as a percentage of control and shown as mean values ± SEM (n=6 per group; n.s). (D) Incorporation of [^{35}S]Met/Cys into eIF2Bε was measured in eIF2Bε immunoprecipitates as described under "Materials and Methods". An autoradiograph from a typical experiment is shown as an inset. Within each experiment, three separate dishes of cells was analyzed per condition, and three such experiments were performed. * $p<0.05$ *vs.* control. (E) Rat 2 fibroblasts transfected with either the pRK7 or FLAG-Rheb plasmid were serum starved as described in Panel A and rapamycin was added to one-half of the culture dishes as described in the legend to Fig. 3. Rheb expression and S6K1 phosphorylation were assessed by Western blot analysis. Blots representative of 3 independent experiments are shown. (F) RNA from the each condition depicted in Panel E was subjected to sucrose density gradient centrifugation followed by isolation of RNA from the subpolysomal and polysomal fractions. Total RNA was analyzed by qRT-PCR as described in Panel C. Within each experiment, three separate dishes of cells was analyzed per condition, and three such experiments were performed. Relative values for eIF2Bε were normalized to GAPDH, expressed as a percentage of control and shown as mean values ± SEM. * $p<0.01$ *vs.* control

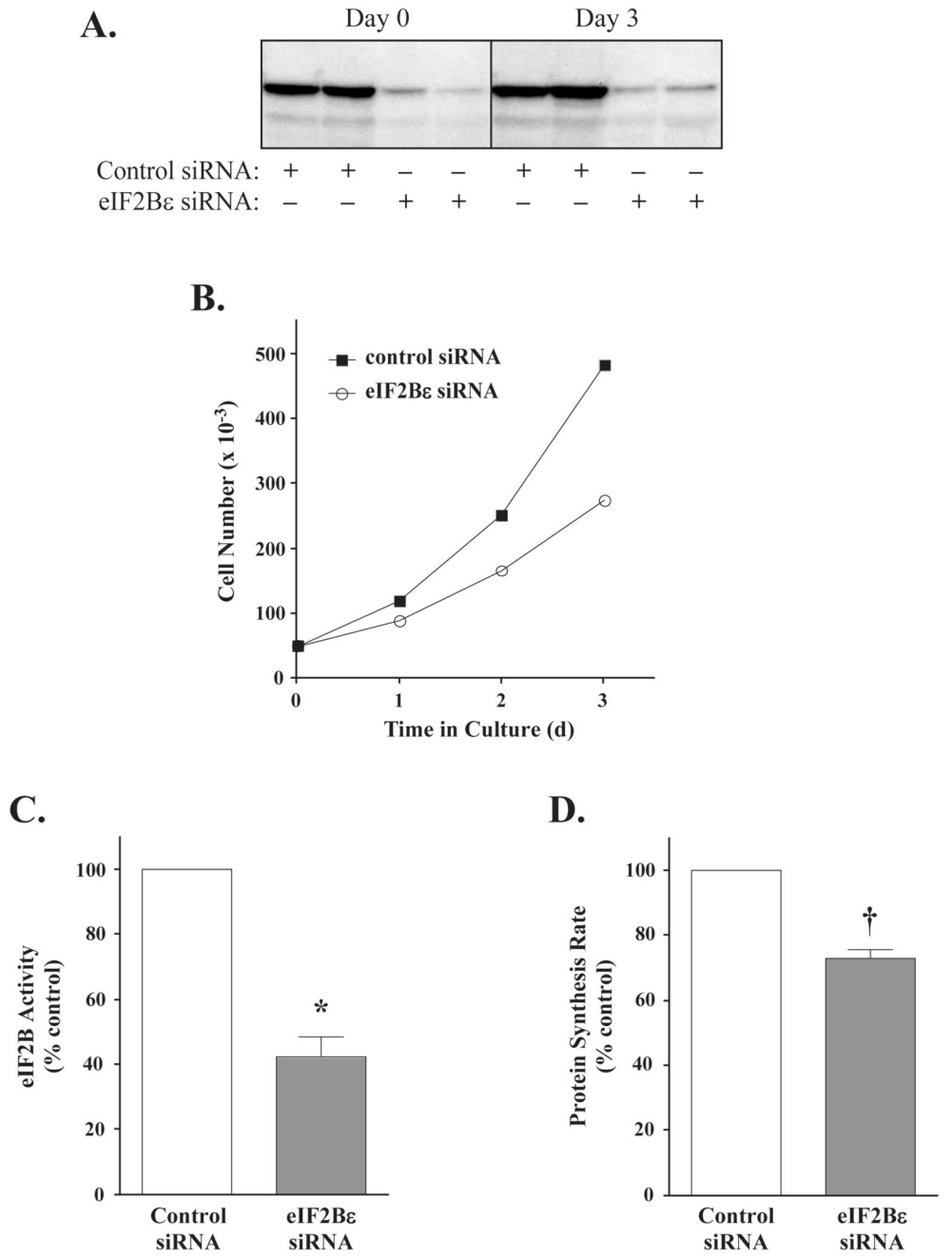

Fig 5.

Decreased eIF2Bε expression leads to reduced cellular proliferation rate, eIF2B activity, and global rates of protein synthesis. Rat 2 cells at 50% confluency were transfected with siRNA targeted against eIF2Bε or a scrambled (control) siRNA as described under “Materials and Methods”. Twenty-four hours later, 5×10^4 cells were equally plated into 100mm dishes. (A) Protein lysates were prepared from cells at 0 and 72h after plating and 50 μg of protein was subjected to Western blot analysis using anti-eIF2Bε antibodies. (B) eIF2B guanine nucleotide exchange activity was measured as described under “Materials and Methods" and is expressed as a percentage of the activity observed in homogenates of cells transfected with the control siRNA. Within each experiment, three separate dishes of cells were analyzed per condition,

and three such experiments were performed. (C) Global rates of protein synthesis were measured as described under “Materials and Methods” and are expressed as a percentage of the rate observed in cells transfected with the control siRNA. Within each experiment, three separate dishes of cells were analyzed per condition, and seven such experiments were performed. * $p<0.05$ *vs* control; † $p<0.01$ *vs* control. (D) For the following 72 hrs, cells were counted at 24 hr intervals and are compiled from 3 separate experiments with 0 hr being the time of plating.