Blocking eukaryotic initiation factor 4F complex formation does not inhibit the mTORC1-dependent activation of protein synthesis in cardiomyocytes

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Huang BP, Wang Y, Wang X, Wang Z, Proud CG. Blocking eukaryotic initiation factor 4F complex formation does not inhibit the mTORC1-dependent activation of protein synthesis in cardiomyocytes. Am J Physiol Heart Circ Physiol 296: H505–H514, 2009. First published December 12, 2008; doi:10.1152/ajpheart.01105.2008.—Activation of the mammalian target of rapamycin complex 1 (mTORC1) causes the dissociation of eukaryotic initiation factor 4E complex (eIF4E)-binding protein 1 (4E-BP1) from eIF4E, leading to increased eIF4F complex formation. mTORC1 positively regulates protein synthesis and is implicated in several diseases including cardiac hypertrophy, a potentially fatal disorder involving increased cardiomyocyte size. The importance of 4E-BP1 in mTORC1-regulated protein synthesis was investigated by overexpressing 4E-BP1, which blocks eIF4F formation in isolated primary cardiomyocytes without affecting other targets for mTORC1 signaling. Interestingly, blocking eIF4F formation did not impair the degree of activation of overall protein synthesis by the hypertrophic agent phenylephrine (PE), which, furthermore, remained dependent on mTORC1. Overexpressing 4E-BP1 also had a small effect on PE-induced cardiomyocyte growth. Overexpressing 4E-BP1 did diminish the PE-stimulated synthesis of luciferase encoded by structured mRNAs, confirming that such mRNAs do require eIF4F for their translation in cardiomyocytes. These data imply that the substantial inhibition of cardiomyocyte protein synthesis and growth caused by inhibiting mTORC1 cannot be attributed to the activation of 4E-BP1 or loss of eIF4F complexes. Our data indicate that increased eIF4F formation plays, at most, only a minor role in the mTORC1-dependent activation of overall protein synthesis in these primary cells but is required for the translation of structured mRNAs. Therefore, other mTORC1 targets are more important in the inhibition by rapamycin of the rapid activation of protein synthesis and of cell growth.

eukaryotic initiation factor 4E-binding protein 1; cardiac hypertrophy; mitochondrial ribonucleic acid translation; rapamycin

**PROTEIN SYNTHESIS** (mRNA translation) is of key importance in cells for their survival, growth, and proliferation. It is an expensive process, requiring both metabolic energy and amino acids, and is therefore under tight control. It is activated by anabolic hormones, by mitogens, and by agents that cause cell or tissue growth (i.e., hypertrophic agents) (16, 31, 46). Conversely, rates of protein synthesis decrease in response to the inadequate availability of energy or amino acids.

Defects in the control of protein synthesis can lead to human disease. For example, cardiac hypertrophy is a major risk factor for heart failure and accounts for a substantial proportion of mortalities among young adults in developed countries (11). The principal factor driving cardiac hypertrophy is a high rate of protein synthesis (16). Therefore, it is important to understand the molecular mechanisms that regulate rates of protein synthesis in heart muscle cells.

Substantial progress has been made in elucidating the mechanisms that control the translational machinery in general. Several of these involve changes in the phosphorylation of translation initiation or elongation factors. However, it is so far unclear what contribution different regulatory mechanisms actually make to controlling overall protein synthesis, particularly in primary cells. One of the key signaling pathways that controls mRNA translation involves the mammalian target of rapamycin mTOR (21, 46, 49). Many of the signaling events controlled by mTOR are acutely blocked by rapamycin, specifically those that are mediated via mTOR complex 1 (mTORC1). Rapamycin and related compounds are in clinical use to prevent graft rejection and cardiovascular disorders such as restenosis after angioplasty (9). Rapamycin analogs are in clinical trials as anti-cancer drugs (5, 7).

Most relevant for the present investigation are the observations that rapamycin can prevent (1, 2, 15, 40) or even reverse (12, 23) overload-induced hypertrophy in animal models (e.g., induced by aortic banding). This shows that mTORC1 signaling is pivotal in the development and maintenance of the hypertrophic state. Our earlier work showed that in isolated cardiomyocytes, the short-term activation of protein synthesis by the hypertrophic agent phenylephrine (PE) and by endothelin-1 was almost completely blocked by rapamycin (44). This indicates that mTOR signaling plays a major role in controlling protein synthesis rates within cardiomyocytes under such conditions. In addition to their relevance for understanding the fundamental mechanisms involved in the mTOR complex 1 (mTORC1)-dependent development of cardiac hypertrophy, cardiomyocytes are a valuable model system for studying the signaling mechanisms that control protein synthesis in differentiated primary cells rather than in the transformed cell lines used in most studies.

mTORC1 is known to control three types of proteins linked to the regulation of the protein synthetic machinery. These include the ribosomal protein S6 kinases, which are activated by mTORC1 signaling (for reviews see Refs. 46 and 49), and phosphorylate ribosomal protein S6. Despite extensive investigation, the function of S6 phosphorylation remains unclear (35). In particular, mice in which both S6 kinase genes have been knocked out still develop cardiac hypertrophy in response to overload or exercise (24), indicating that the S6 kinases do not play an essential role in cardiac hypertrophy or, presumably, in the activation of protein synthesis that underlies it.

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mTORC1 also controls the activity of eukaryotic initiation factor 4E complex (eIF4E), the protein that binds the 5′-terminal cap present on all cytoplasmic mRNAs (which includes a 7-methylguanosine triphosphate moiety; m7GTP). By binding to the scaffold protein eIF4G, eIF4E is involved in assembling translation initiation complexes that recruit the 40S ribosomal subunit to the mRNA (14). The ability of eIF4E to participate in such complexes is blocked by small inhibitory phosphoproteins such as eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), which occlude the binding site of eIF4E for eIF4G. Activation of mTORC1 leads to the hyperphosphorylation of 4E-BP1 and to its release from eIF4E, allowing eIF4E to bind eIF4G and form active initiation complexes. The heterotrimeric complex containing eIF4E, eIF4G, and eIF4A is often referred to as eIF4F (14). eIF4A is an RNA helicase, which can unwind secondary structure in RNA molecules. eIF4E also binds the poly(A)-binding protein, PABP, thereby circularizing the mRNA by bringing its 3′- and 5′-ends into proximity (reviewed in Ref. 36). Such circularization appears to be important for the efficient translation of mRNAs. Much attention has been focused on eIF4E, and its regulation by the 4E-BPs, as a key step in the control of mRNA translation (14, 33). In particular, it is widely thought that eIF4E is the rate-limiting factor for translation initiation and thus that the increased formation of eIF4F complexes elicited by agents that stimulate translation plays an important role in activating protein synthesis (reviewed in Refs. 10 and 13). This could, therefore, provide a mechanism by which signaling through mTORC1 might promote translation initiation. However, the importance of the increased formation of eIF4F complexes in the mTORC1-dependent control of general protein synthesis is poorly understood.

mTORC1 signaling also activates eukaryotic elongation factor 2 (eEF2), which mediates the movement of the ribosome along the mRNA. Phosphorylation of eEF2 at Thr56 by eEF2 kinase causes its inactivation. By bringing about the inactivation of eEF2 kinase, signaling through mTORC1 causes the dephosphorylation and activation of eEF2, thereby activating translation elongation (reviewed in Ref. 18).

The major goal of this study was to assess the importance of the mTORC1-regulated binding of eIF4G to eIF4E in the rapamycin-sensitive activation of protein synthesis, and cell growth, in adult rat ventricular cardiomyocytes (ARVC). Previous studies (6, 28) have assessed the importance of eIF4F for protein synthesis by either cleaving it using a viral protease (e.g., to separate the eIF4E- and eIF4A-binding sites) or by knocking down its expression using small interfering RNA. However, these approaches assess the requirement for (intact) eIF4G. In contrast, the aim of this study was to explore specifically the importance of the mTORC1-regulated eIF4E:eIF4G interaction in the mTORC1-dependent activation of protein synthesis. This distinction is important given that eIF4G interacts, via binding sites distributed across its length, with multiple components of the translational components (e.g., PABP, eIF4A, eIF3, eIF4B, and the eIF4E kinases the Mnks) (14, 30). Thus knockdown or cleavage of eIF4G will have different and likely more widespread effects than simply blocking the eIF4E:eIF4G interaction.

EXPERIMENTAL PROCEDURES

Chemicals, antisera, and other materials. L-[35S]methionine, m7GTP-Septahose CL-4B, protein G Sepharose, and enhanced chemiluminescence (ECL) reagents were purchased from Amersham Pharmacia Biotech. Rapamycin was acquired from Calbiochem. BSA (fraction V) was from Roche Molecular Biochemicals. Collagenase (type II) was purchased from Worthington, NJ. Anti-eIF4G antibodies were kindly provided by Dr. Simon Morley (Sussex, UK). Anti-phospho-Ser235/6 ribosomal protein S6, anti-ribosomal protein S6, and anti-phospho-Ser64 4E-BP1 antibodies were purchased from Cell Signaling Technology. Anti-4E-BP1, anti-eEF2, and anti-phosphoThr56 eEF2 antibodies (the numbering system takes account of the removal of the N-terminal methionine of eEF2) were prepared as previously described (4). The luciferase B0 and B4 adenoviruses were kind gifts from Dr. Paul McDermott (42). 4EGI-1 (26) was a generous gift from Drs. Gerhard Wagner and Ricard Rodriguez (Harvard Medical School). All other chemicals or biochemicals were obtained from Sigma-Aldrich unless otherwise stated.

Isolation, culture, treatment, and lysis of cardiomyocytes. ARVC were prepared from adult male Sprague-Dawley rats (250–300 g; Animal Care Center, University of British Columbia) by collagenase perfusion (45). After isolation, cells were washed, seeded onto laminin-coated tissue culture dishes, and allowed to attach for 2 h at 37°C. Attached cells were cultured in modified M199 medium containing 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 5 mM creatine, 2 mM carnitine, 5 mM taurine, and 1.0 mM EGTA for 40 h before further treatment. After treatments, as indicated in the figure legends, ARVC were lysed by scraping with extraction buffer containing 50 mM Tris (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1 mM Na3VO4, 50 mM NaF, 5 mM sodium pyrophosphate, 1% (vol/vol) Triton X-100, 1 mM dithiotreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml antipain, and 0.2 mM phenylmethyl sulphonyl fluoride. Protein concentrations were determined as described (3).

Animals were housed and euthanized in strict accordance with the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals (volume 1, 1993). This work was approved by the local (University of British Columbia) Animal Care Committee.

Construction and use of adenoviral vectors. The aden-X Expression System (BD Biosciences) was used to construct all the adenoviruses, strictly as described in the instruction manual [except the green fluorescent protein (GFP) virus used previously (34), which was made using the AdEasy system]. In addition, gel purification was utilized to purify DNA fragments after restriction digestion of the pShuttle vector.

Adenovirus-mediated gene transfer was performed after ARVC had attached to the laminin-coated tissue culture dishes (44, 45). ARVC were incubated at 37°C with recombinant adenoviruses in minimal volume of M199 medium for 2 to 3 h at the multiplicity of infection (moi), indicated in the figure legends. Fresh M199 medium was supplied to the cells after removing the virus. ARVC were cultured for an additional 40 h to allow expression of the genes of interest before further treatment. Viruses encoding either the GFP or β-galactosidase (LacZ) were used as negative controls. The former allows the efficiency of transduction to be assessed readily under the fluorescence microscope. Since the LacZ virus was constructed using the BD Adeno-X Expression System employed to create the vectors for wild-type (WT) and mutant 4E-BP1 (whereas the GFP virus was not), it was used as the control in many of the experiments described here. In fact, neither of these control viruses had affected the parameters studied here, and they can therefore be used interchangeably. In particular, adenovirus transduction does not activate either Akt (protein kinase B, which is stimulated through phosphorylation of 3-kinase) or ERK signaling (48), pathways that can activate the protein synthetic machinery.

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**RESULTS**

Overexpression of 4E-BP1 blocks formation of eIF4E/eIF4G complexes in ARVC. We have previously shown that the short-term (2 h) treatment of ARVC with the hypertrophic agent PE elicits a substantial stimulation of protein synthesis, which is largely blocked by rapamycin (44). This indicated a key role for signaling through mTORC1 in stimulating protein synthesis and provided a physiologically relevant primary cell system for analyzing which component(s) downstream of mTORC1 is/are involved in the activation of protein synthesis. The best-studied targets of mTORC1 are the S6 kinases and the 4E-BPs. As mentioned in Experimental procedures, data from S6 kinase 1/S6 kinase 2 double knockout mice revealed that these enzymes are not required for cardiac hypertrophy in response to work overload or exercise (24).

Accordingly, we focused our attention on 4E-BP1 and the formation of eIF4E/eIF4G complexes, since it is widely believed that they play a major role in the control of protein synthesis, as described in Experimental procedures. One possible approach to assessing the importance of the eIF4E/eIF4G interaction for the activation of protein synthesis would be to use cell-permeant peptides based on the shared eIF4E-binding motif in eIF4G and 4E-BP1 (Fig. 1A) (17). However, our earlier data showed that such peptides rapidly induced cell death in several cell types, and we have accordingly avoided using them here.

To address the importance of the mTOR-regulated formation of eIF4F complexes in the control of protein synthesis in ARVC, we therefore adopted an alternative approach. We created adenoviral vectors encoding WT 4E-BP1 or a mutant in which the eIF4E binding site has been mutated to render the protein unable to bind eIF4E, as a negative control. For the latter, we introduced two mutations into the common binding motif shared by 4E-BPs and eIF4G, thus creating the L58A/M59A (LM/AA) mutant (Fig. 1A). We preferred not to use a nonphosphorylatable mutant of 4E-BP1 (in which certain phosphorylation sites are mutated to alanines), since such mutants are reported to bind rather stably to the mTORC1 component raptor and would likely interfere with other signaling events downstream of mTORC1 (8), making it difficult to interpret the resulting data.

ARVC were transduced with adenoviruses encoding the WT or LM/AA forms of 4E-BP1 or, as a further control, GFP. In initial experiments, we conducted a titration of the viruses to assess the relative levels of overexpression of WT 4E-BP1 and the LM/AA mutant (data not shown). As can be seen in Fig. 1B, using these viruses at 25 and 50 moi, respectively, yielded similar levels of overexpression as judged by subjecting cell lysates to Western blot using anti-myc. Using higher levels of the LM/AA virus did not achieve significantly higher levels of overexpression of the mutant 4E-BP1. Therefore, in subsequent experiments we used the WT virus at 25 moi and the LM/AA and GFP control each at 50 moi, unless indicated otherwise (see figures). Cells overexpressing 4E-BP1 did not show any morphological changes (data not shown).

In some cases cells were treated with PE. As expected from earlier data (44), PE induced the release of endogenous 4E-BP1 from eIF4E and increased the binding of eIF4G to eIF4E (Fig. 1C) in the GFP-expressing cells (data for the ratio of the signals eIF4G and eIF4E for a typical experiment are presented below each lane). Overexpressed WT 4E-BP1 bound to eIF4E under both conditions (±PE) and was not released in response to PE. Consistent with this, the overexpression of 4E-BP1 reproducibly blocked both basal and PE-induced eIF4G binding (this was observed in each of 10 independent experiments).
In cells overexpressing the nonbinding LM/AA mutant (which, as expected, did not copurify with eIF4E; Fig. 1C, but here cytosolic lysates were subjected to affinity chromatography on m7GTP-Sepharose. The bound material was analyzed by SDS-PAGE/immunoblotting using the indicated antibodies. In the bottom section, the positions of the endogenous (Endog) and the ectopically expressed 4E-BP1 polypeptides are shown. Numbers show the relative eIF4G-to-eIF4E ratios as an average of 3 independent experiments ± SE. D: ARVC were transduced with the indicated adenoviral vectors and treated with PE as described for B. Samples of cell lysate were analyzed by SDS-PAGE and Western blot with the indicated antibodies. In all cases, the data shown are representative of findings from at least 3 independent experiments. eEF2, eukaryotic elongation factor 2; Exog, exogenous; Endog., endogenous; moi, multiplicity of infection.

Fig. 1. Eukaryotic initiation factor 4e-binding protein 1 (4E-BP1) blocks eukaryotic initiation factor 4G complex (eIF4G)/eukaryotic initiation factor 4E complex (eIF4E) binding in adult rat ventricular cardiomyocytes (ARVC). A: eIF4E-binding motifs of 4E-BP1 and eIF4G are shown, together with the modified sequence in the L58A/M59A (LM/AA) mutant used here. Residues known to be important for the binding of 4E-BP1 or eIF4G to eIF4E are underlined. B: ARVC were transduced with adenoviruses encoding green fluorescent protein (GFP; negative control protein), myc-tagged wild-type (WT) 4E-BP1, or the LM/AA mutant of 4E-BP1. Forty hours later, some plates were treated with phenoxyphrine (PE; 10 μM, 60 min) as indicated. Cell lysates were analyzed directly by SDS-PAGE/immunoblot using the indicated antisera. For endogenous 4E-BP1, the α-, β-, and γ-species of 4E-BP1 are shown (α-species being the least phosphorylated and γ-species the most). Note that only the hyperphosphorylated (γ) form of 4E-BP1 is phosphorylated at Ser64. Blots were also probed with anti-myc to detect ectopically expressed 4E-BP1; as a consequence of the tag, this protein only shows a limited mobility shift upon phosphorylation (arrowheads).

C: a si
B
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In cells overexpressing the nonbinding LM/AA mutant (which, as expected, did not copurify with eIF4E; Fig. 1C), the levels of eIF4G bound to eIF4E were similar after PE treatment to those seen in the GFP-expressing cells (Fig. 1C; data are quantified below the panel). In each of 10 independent experiments performed, WT 4E-BP1 blocked both basal and PE-induced binding of eIF4G to eIF4E. As shown in Fig. 1B, both the endogenous and ectopically expressed 4E-BP1 did undergo phosphorylation at Ser64 in response to PE. However, the degree of phosphorylation of the overexpressed protein is evidently insufficient to bring about the release of all the 4E-BP1 molecules from eIF4E, since eIF4E remains associated with 4E-BP1 and eIF4G binding is blocked, even after treatment of the cells with PE. This may arise because the total amount of 4E-BP1 exceeds the capacity of the cells to phosphorylate it all. Other explanations are possible.

The reason for conducting these experiments was to assess the role of PE-induced eIF4G/eIF4E binding (eIF4F formation) in the activation of protein synthesis by PE. The above data provide a strong basis for doing so, but it was crucial to check whether the high-level expression of 4E-BP1 interfered with signaling downstream of mTORC1. One reason this could happen is that the TOR signaling motif in the ectopically expressed 4E-BP1 might bind and titrate out the raptor component of mTORC1 (38), perhaps impeding the regulation of other targets of this complex, such as the S6 kinases and eEF2 kinase (as suggested in Ref. 43). Therefore, we studied the phosphorylation of these proteins in ARVC expressing GFP or 4E-BP1 (Fig. 1D). To examine whether the overexpression of 4E-BP1 affected the ability of PE to activate mTORC1 signaling, we studied the phosphorylation of Ser235/6 and Ser240/4 in S6, Ser64 in 4E-BP1, and Thr56 in eEF2. (Ser240/4 in S6 were studied since their phosphorylation is completely dependent on the S6 kinases, unlike that of Ser235/6, which is not.) Other kinases such as p90RSK may play in phosphorylating (29), although in these experiments rapamycin did block the
basal phosphorylation of S6 at all sites tested, suggesting that p90RSK actually plays little role in their phosphorylation under these conditions (data not shown), which were very similar regardless of whether ARVC overexpressed WT or LM/AA 4E-BP1 or GFP. In contrast, both effects were, as expected (44), completely blocked by rapamycin (data not shown). Thus overexpressing 4E-BP1 does not impair mTORC1 signaling.

In fact, overexpressing WT 4E-BP1 actually tended to enhance the basal phosphorylation of the endogenous 4E-BP1 (Fig. 1B). Interestingly, the LM/AA mutant did not exert this effect. This phenomenon may be related to the effects described in Ref. 19, which reported, conversely, that the overexpression of eIF4E decreased 4E-BP1 phosphorylation. The mechanism underlying this effect remains unclear. Since overexpressing 4E-BP1 in ARVC did not affect the phosphorylation of other targets of mTORC1, i.e., S6 and eEF2 (Fig. 1D), the effect seems to be specific for 4E-BPs and, taking into account the data of Ref. 19, appears to be linked to the activation state of eIF4E.

**Overexpression of 4E-BP1 does not prevent the activation of protein synthesis by PE.** To assess the effect of PE on rates of protein synthesis, we examined the incorporation of labeled amino acid ([35S]methionine) into TCA-precipitable material; in a similar way to our previous work (44, 45), we studied this over the shortest feasible time periods (90 min of PE treatment followed by 45 of radiolabeling). This is to minimize potential contributions from changes, e.g., in transcription and/or ribosome biogenesis. Overexpression of WT 4E-BP1 partially inhibited the basal rate of protein synthesis in ARVC (Fig. 2A). This clearly is not due to any impairment of mTORC1 signaling (Fig. 1D) and more likely reflects the loss of the basal level of eIF4F complexes (Fig. 1C). This conclusion is reinforced by the observation that overexpressing the LM/AA mutant (which was present at similar levels to the WT protein; data not shown; Fig. 1B) did not affect basal protein synthesis rates (Fig. 2B). This is consistent with the notion that the reduced basal rate of protein synthesis is due to the loss eIF4F and related initiation complexes. This might, in part, account for the fact that rapamycin decreases basal protein synthesis rates (Fig. 2A).

To assess the importance of the induction of eIF4E/eIF4G binding for the activation of protein synthesis by PE, we treated ARVC overexpressing GFP or WT 4E-BP1 with PE and measured the rate of protein synthesis. Short-term (~2 h) treatment with PE markedly activated protein synthesis in the GFP-expressing cells (Fig. 2A). When the lower basal rate of protein synthesis in cells overexpressing ectopic 4E-BP1 is taken into account, PE activated protein synthesis at least as strongly in these cells as in the controls. Indeed, the percent increase in protein synthesis induced by PE was actually greater in the 4E-BP1-expressing cells (increase of 80% in control cells and 107% in cells overexpressing WT 4E-BP1; difference is significant: P < 0.05). This could, in principle, reflect a role for mTORC1-independent regulators of protein synthesis. However, this possibility is ruled out by studying the phosphorylation of S6; longer exposure revealed detectable basal phosphorylation of S6, which was blocked by rapamycin (not shown). The fact that PE still markedly activates protein synthesis in cells that are overexpressing WT 4E-BP1 indicates that cell viability is not adversely affected.

These data lead to two important conclusions. First, the increased formation of eIF4F is not required for the acute activation of protein synthesis by PE. Finally, since the PE-induced activation of protein synthesis remains sensitive to rapamycin, it must involve other events that lie downstream of mTORC1 that contribute to the short-term activation of protein synthesis. [Since we are studying the short-term effects of PE, changes in ribosome content, which can also be regulated by mTORC1 (22), may essentially be disregarded over the time frame of this analysis.] To confirm and extend these unexpected findings, we analyzed newly synthesized proteins in ARVC by SDS-PAGE and fluorography. Since this requires increased levels of radiola-

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**Fig. 2. 4E-BP1 does not block the ability of PE to activate protein synthesis in ARVC.** A and B: ARVC were transduced with adenoviruses encoding GFP or myc-tagged 4E-BP1 (and in B the LM/AA mutant). In A, 40 h later, some plates were treated with PE (10 μM, 90 min) and/or treated with rapamycin (Rap; 100 nM, added 30 min before PE stimulation). L-[35S]methionine (5 μCi/ml) was then added to the medium, and 45 min later cells were washed with ice-cold PBS and lysed. Data are corrected for the protein content of the lysate and are normalized to the level of protein synthesis in untreated cells expressing GFP. Data are presented as mean values ± SE for 3 independent experiments. P < 0.05 *vs. GFP control (ctrl); #vs. 4E-BP1 control; ¤vs. GFP cells treated with PE; ¶vs. 4E-BP1-overexpressing cells treated with PE. A, inset: Western blot for S6 phosphorylated at Ser235/236 and a loading control for total S6 levels, which are representative of the 3 experiments conducted. B shows the basal rates of protein synthesis in cells 40 h after transduction with the indicated viruses.
beling, we modified the procedure in two ways: 1) we used methionine-free medium (to increase the effective specific radioactivity of the labeled methionine) and 2) we labeled for a longer period. Pilot experiments revealed that a 6-h labeling period was the shortest feasible time period. In some cases, the translation elongation inhibitor cycloheximide (CHX) was added to the cells before the addition of labeled methionine. As shown in Fig. 3A, CHX almost completely blocked the labeling of all the visible bands, confirming that the incorporation of label is indeed due to protein synthesis rather than any other fate of the label. It is also clear from Fig. 3A that the general pattern of labeled polypeptides is similar in ARVC overexpressing 4E-BP1 or LacZ. (The β-galactosidase band is missing from the 4E-BP1-expressing samples since these cells were not transduced with the LacZ virus.) These data strongly imply that the loss of eIF4F complexes is not accompanied by a gross shift in protein synthesis toward different mRNAs, which might be translated in a cap-independent manner, e.g., driven by internal ribosome-entry segments. Given their high abundance in myocytes, one might expect to see prominent signals for actin (40 kDa) and myosin (>200 kDa). One of the strong bands around 40 kDa may be actin. Its relative rate of synthesis is about half that of ventricular proteins in general (25). Actin and myosin may be underrepresented on the gel due to relatively poor extraction under the low salt conditions used here (as compared with cytosolic proteins) and, in the case of myosin, perhaps also due to its large size. There is no reason to believe that the availability of eIF4F complexes would exert a differential effect on the regulation of short-lived cytosolic versus longer lived or less soluble proteins.

As shown in Fig. 3B, PE enhanced the general incorporation of label into all the detectable proteins in 4E-BP1-expressing cells, and this increase was inhibited by rapamycin. These findings confirm that the data for amino acid incorporation shown in Fig. 2A do truly reflect changes in the rates of synthesis of a broad range of polypeptides. As in Fig. 3A, no differences in the synthesis of any specific polypeptides were observed in this analysis (Fig. 3B).

Taken together, these findings indicate that, in the short term, 4E-BP1 does not affect the synthesis of any of the abundant proteins observed using this approach. The inhibition of basal rates of translation in 4E-BP1-expressing cells therefore reflects a rather general effect, although it is clearly possible that 4E-BP1 does more markedly impair the translation of lower abundance mRNAs whose translated products are not seen in this analysis.

4E-BP1 does not block PE-induced heart cell growth. Since increased rates of protein synthesis are the main factors that drive hypertrophy of cardiomyocytes (16), it was important to study whether 4E-BP1 affected the induction of cell growth by the hypertrophic agonist PE. To assess cell growth in different treatments, we measured the area of ARVC, an approach that is commonly employed to estimate cardiomyocyte growth (for two examples, please see Refs. 32 and 50). Our recent work indicates that measuring cell area accurately reflects changes in cell volume and, therefore, overall cell size (rather than reflecting spreading of the cells on the laminin substratum) (48). PE increased the size of ARVC, and this change was entirely blocked by rapamycin, indicating that it is completely dependent upon signaling through mTORC1 (48).
only exerted a small (but statistically significant, \( P < 0.05 \)) inhibitory effect on PE-induced myocyte growth. The substantial residual growth was completely inhibited by rapamycin, indicating that it remains entirely dependent upon mTORC1 signaling (Fig. 4). eIF4F complexes are therefore dispensable for the induction of cell growth by the hypertrophic stimulus PE. Thus the activation of cell growth and protein synthesis by PE, respectively, are largely and almost entirely independent of the formation of eIF4F complexes.

**4E-BP1 suppresses the PE-induced activation of the translation of structured mRNAs.** It was possible that small and undetectable residual amounts of eIF4F might suffice to support the activation of protein synthesis. eIF4F is thought to be particularly important for the translation of mRNAs with structured 5′-UTRs. The eIF4A component of eIF4F possesses RNA helicase activity, which can unravel secondary structure in the 5′-UTR of the mRNA to facilitate the initiation of translation (depicted in Fig. 5A) (30). Therefore, we extended our study to examine the effect of overexpressing 4E-BP1 on specific mRNAs that contain substantial secondary structure, using the adenoviral luciferase reporter system created by the McDermott laboratory (42). This includes viruses that encode the luciferase reporter downstream either of a fairly structured 5′-UTR (termed the B0 virus, predicted \( \Delta G = -57 \text{ kcal/mol} \)) or of a 5′-UTR with more extensive secondary structure (B4; containing 4 contiguous BamH1 inserts, predicted \( \Delta G = -112 \text{ kcal/mol} \)). To allow 4E-BP1 to be made before the expression of the luciferase reporters, ARVC were first transduced with adenoviruses encoding LacZ and WT 4E-BP1. Sixteen hours later, ARVC were further transduced with the luciferase viruses and then treated with PE. Twenty hours later, cells were lysed and luciferase assays were performed. Care was taken to ensure that all assays were within the linear range and to determine the expression levels of the luciferase mRNAs (by quantitative RT-PCR). The data in Fig. 5B are normalized for the levels of luciferase mRNA expression in each case.

Under unstimulated conditions (no PE), the expression of luciferase from the B4 vector was somewhat lower than that from the less structured B0 vector, as expected since the former contains more secondary structure in its 5′-UTR (Fig. 5B). Treatment of ARVC with PE increased luciferase expression from either vector to rather similar extents in control cells (B0, 1,424 ± 46% of unstimulated control; and B4, 1,172 ± 67%). The observation that PE markedly enhances the synthesis of structured mRNAs is consistent with its ability to promote the formation of eIF4F complexes, which are considered to be especially important for the translation of such messages. The additional secondary structure in the B4 mRNA is clearly efficiently overcome after PE stimulation of control cells, since PE stimulates the synthesis of luciferase from both constructs to rather similar extents from both mRNAs are similar. The effect of PE on luciferase expression is greater than that on total protein synthesis likely because, under basal conditions in particular, the secondary structure impedes the translation of both the B0- and B4-derived mRNAs.

Overexpression of 4E-BP1 substantially blunted the PE-stimulated expression of luciferase from the B0 vector and completely blocked PE-enhanced B4 luciferase expression (Fig. 5B). The fact that PE was unable to stimulate luciferase expression from the B4 vector under a condition where eIF4F formation is blocked suggests that although general mRNA translation is stimulated by a mechanism(s) that is distinct from enhancement of eIF4F formation (as concluded above on the basis of the data for general protein synthesis in Fig. 2), specific mRNAs, such as those with structured 5′-UTRs, do require eIF4F for their efficient translation in ARVC. These data clearly distinguish between the stimulation of overall protein synthesis, which is hardly affected by the overexpression of 4E-BP1, and the expression of the structured reporters, for which PE-induced stimulation is either substantially blocked or abolished.

**DISCUSSION**

The goal of this study was to investigate the importance of the PE-induced, rapamycin-sensitive binding of eIF4E to eIF4F for the mTORC1-dependent activation of protein synthesis in ARVC. To address this, we have overexpressed WT 4E-BP1 in ARVC; this prevents eIF4E from binding to eIF4G to the extent that both the basal and PE-induced formation of
eIF4F complexes is eliminated and studied the effect of this on protein synthesis. The data obtained using this approach lead to three important conclusions. First, loss of eIF4F complexes somewhat decreases, but does not eliminate, the basal rate of protein synthesis. This is consistent with the idea that eIF4F complexes are not essential for the reinitiation events, which maintain ongoing mRNA translation, consonant with earlier conclusions that showed that intact eIF4F was also not necessary for this (28). Second, and more surprisingly, PE activates protein synthesis to at least the same degree in the absence of detectable eIF4F complexes as it does under control conditions. This finding substantially extends earlier work (28) by addressing the role of eIF4F formation in the mTORC1-dependent activation of protein synthesis. Our data show that neither eIF4F complexes nor their increased formation are required for the activation of protein synthesis in ARVC. Finally, the stimulation of protein synthesis in cells overexpressing 4E-BP1 remains strongly sensitive to inhibition by rapamycin. The latter two points demonstrate the importance of other rapamycin-sensitive, and therefore mTORC1-mediated, effects in the activation of protein synthesis.

It has previously been shown using adult feline cardiomyocytes (37) that enhanced overexpression of eIF4E did not by itself stimulate the overall rate of protein synthesis, although it did increase the levels of eIF4E/eIF4G complexes. This indicates that increasing the level of eIF4F is by itself insufficient to enhance protein synthesis rates, consistent with our findings. Importantly, our data extend those studies by demonstrating that increased formation of eIF4F is not required for the mTORC1-dependent activation of protein synthesis. Adenovirus-mediated overexpression of eIF4E also failed to stimulate protein synthesis in ARVC (Huang BP, unpublished observation). Therefore, increased formation of eIF4F seems to be neither necessary nor sufficient for the activation of protein synthesis in primary cardiomyocytes. We have recently reported a similar lack of effect of eIF4E overexpression on protein synthesis in human embryonic kidney 293 cells (47). 4E-BP1 is the major 4E-BP isoform expressed in heart. However, knocking out 4E-BP1 (alone or together with 4E-BP2) in mice did not affect heart size significantly under normal conditions (20, 41). Our data substantially extend those observations by showing that enhanced formation of eIF4F is not actually required for the short-term activation of protein synthesis by the hypertrophic agent PE. Indeed, the loss of detectable basal levels of eIF4F did not decrease the extent to which PE activates protein synthesis. These observations are surprising given the rather widely held view that the (mTORC1-dependent) formation of eIF4F complexes plays an important role in turning on protein synthesis by facilitating translation initiation. It may be that the stimulation of protein synthesis by PE mainly reflects increased translation of mRNAs that are already engaged in polysomes rather than the recruitment of ribosomes to new mRNAs. This would be consistent with the finding that ongoing translation does not require intact eIF4F (28). Nonetheless the fact that overexpression of WT 4E-BP1, but not the (inactive) LM/AA mutant, decreases basal rates of protein synthesis points to a role for eIF4F in maintaining basal mRNA translation.

It is possible that eliminating eIF4F might affect the translation of some endogenous mRNAs more strongly than others (e.g., mRNAs with highly structured 5'UTRs, as shown by our data for reporter constructs), even though it did not impair the activation by PE of overall protein synthetic rates. This idea is fully borne out by the observation that overexpressing 4E-BP1 markedly blunts the ability of PE to enhance the expression of luciferase from mRNAs with modest (B0) or extensive (B4) structure in their 5'-UTRs. Thus it remains entirely possible that eIF4F complexes are important for the stimulation of the translation of certain specific, but relatively low abundance, mRNAs, e.g., with structured 5'-UTRs, even though the formation of eIF4F complexes does not play a major role in the rapamycin-sensitive activation of general protein synthesis in ARVC.

As an adjunct to the above studies, we also attempted to use a recently described inhibitor of the eIF4E:eIF4G interaction: 4EGI-1 (26). 4EGI-1 did very effectively block eIF4E:eIF4G binding in ARVC (supplemental Fig. 1A; all supplemental material can be found with the online version of this article). However, this compound also resulted in a very rapid (10–60 min) and marked decrease in cell viability (supplemental Fig. 1B). This contrasts with overexpressing 4E-BP1, which did not affect cell viability. This rapid loss of cell viability precluded using this compound to assess the importance of eIF4E:eIF4G complexes in the control of protein synthesis. A similar effect was reported in the first study using this compound, although for the (Jurkat) cells used there, only longer exposure to 4EGI-1 induced cell death (26). The finding that 4EGI-1 leads to rapid death of ARVC is in agreement with our earlier work in which a cell-permeant peptide that binds to eIF4E and blocks eIF4G binding also rapidly induced cell death (17). The reasons for this marked difference between the effects on cell survival of different ways of inhibiting eIF4E:eIF4G binding are not clear and merit further investigation, which lies beyond the scope of this study.

It is clearly now very important to identify which mTORC1-regulated translation components do contribute to the mTORC1-dependent stimulation of general mRNA translation. As discussed above, this is unlikely to involve the S6 kinases (24) but could, for example, involve the mTORC1-dependent control of eEF2 (18), a protein that is required for the elongation stage of the translation of all mRNAs.

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