Modifications of eukaryotic initiation factor 4F (eIF4F) in adult cardiocytes by adenoviral gene transfer: differential effects on eIF4F activity and total protein synthesis rates

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In adult feline cardiocytes, increases in eukaryotic initiation factor 4F (eIF4F) activity are correlated with accelerated rates of total protein synthesis produced in response to increased load. Adenoviral gene transfer was employed to increase either eIF4F complex formation or the phosphorylation of eIF4E on Ser-209. To simulate load, cardiocytes were electrically stimulated to contract (2 Hz, 5 ms pulses). Non-stimulated cardiocytes were used as controls. Adenovirus-mediated overexpression of wild-type eIF4E increased the total eIF4E pool by 120–140% above endogenous levels after 24 h and produced a corresponding increase in eIF4F content. However, it did not accelerate total protein synthesis rates in quiescent cardiocytes; neither did it potentiate the increase produced by contraction. To modify the affinity of eIF4F, cardiocytes were infected with a mutant (eIF4E/W56F) with a decreased binding affinity for the mRNA cap. Overexpression of eIF4E/W56F increased the quantity of

eIF4F but the rate of total protein synthesis was decreased in quiescent and contracting cardiocytes. Overexpression of a mutant that blocked eIF4E phosphorylation (eIF4E/S209A) increased the quantity of eIF4F without any significant effect on total protein synthesis rates in quiescent or contracting cardiocytes. Overexpression of the eIF4E kinase Mnk-1 increased eIF4E phosphorylation without a corresponding increase in eIF4F complex formation or in the rate of total protein synthesis. We conclude the following: (1) eIF4F assembly is increased by raising eIF4E levels via adenoviral gene transfer; (2) the cap binding affinity of eIF4F is a rate-limiting determinant for total protein synthesis rates; and (3) increases in the quantity of eIF4F alone or in eIF4E phosphorylation are not sufficient to accelerate total protein synthesis rates.

Key words: adenovirus, hypertrophy, translation.

INTRODUCTION

The adult heart compensates for haemodynamic overload by undergoing hypertrophic growth, whereby an increase in left ventricle mass normalizes myocardial wall stress within a normal range [1–3]. During hypertrophy, accelerating the steady-state rate of protein synthesis by approx. 25–50% produces a net accumulation of constitutively expressed proteins in the cardiocyte such as myofibrillar and mitochondrial proteins [4,5]. There are two general translation mechanisms that accelerate the rate of protein synthesis, efficiency and capacity. Efficiency is defined as the utilization of the mRNA pool by the existent translational machinery and is calculated by dividing the rate of total protein synthesis by ribosome content [4]. Capacity refers to the overall amount of translational machinery (ribosomes, tRNA and translation factors) and is usually calculated on the basis of total RNA content. These mechanisms are co-ordinately regulated during pressure-overload hypertrophy *in vivo*, as indicated by an initial increase in efficiency that is followed by an adaptive increase in capacity [6–8]. Given that the total mRNA pool is not rate-limiting for protein synthesis and that individual mRNA species compete for a limited quantity of ribosomes to initiate

translation [9], changes in the activity and quantity of the translational machinery are essential for accelerating and maintaining the steady-state rates of protein synthesis in the terminally differentiated adult cardiocyte [4].

Translation is controlled at several steps of the initiation phase [10,11]. The first steps involve the dissociation of the ribosomal subunits and formation of the ternary complex. The 40 S ribosomal subunit binds to the ternary complex to form the 43 S pre-initiation complex. This is followed by the binding of mRNA to the 43 S pre-initiation complex to form the 48 S pre-initiation complex. This step is catalysed by the eukaryotic initiation factor 4F (eIF4F) complex, which consists of three proteins: eIF4E, which binds to the 7-methylguanosine (7m-Gppp) cap of the mRNA, eIF4A, which functions as an ATP-dependent RNA helicase that unwinds mRNA secondary structure, and eIF4G, which is a scaffolding protein that contains binding sites for eIF4E and eIF4A. In addition, eIF4G binds to accessory proteins such as poly(A)-binding protein (Pab1p) and Mnk-1 and Mnk-2 [mitogen-activated protein (MAP) kinase signal-integrating kinases], which are thought to modify the cap binding activity of eIF4F [12,13]. Current models of cap-dependent translation indicate that the eIF4F complex binds to the 7m-Gppp cap of

Abbreviations used: eIF, eukaryotic initiation factor; 4E-BP, eIF4E-binding protein; 7m-Gppp, 7-methylguanosine cap; Mnk-1, MAP kinase signal-integrating kinase; HA, influenza haemagglutinin epitope.

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mRNA, which facilitates the unwinding of mRNA secondary structure via the helicase activity of eIF4A and permits the binding of mRNA to 40 S ribosomal subunits to initiate translation (reviewed in [11]).

eIF4F activity is determined by the affinity for the 5'-mGppp cap and by the quantity of eIF4F complexes. At least two mechanisms regulate eIF4F affinity: an increase in intrinsic cap binding activity of eIF4E when it associates with eIF4G [14], and the phosphorylation of eIF4E on Ser-209 by Mnk-1 or Mnk-2, which is postulated to enhance cap binding affinity [12,15]. Several mechanisms might regulate the assembly of eIF4F complexes. First, eIF4E is the least abundant of the 4F proteins and is thought to be a limiting factor in eIF4F assembly [16]. Secondly, the pool of eIF4E available for assembly is regulated by a family of eIF4E-binding proteins (4E-BPs or PHAS-I). They function as translational repressors by competing with eIF4G for a common binding site on eIF4E [17,18]. Thirdly, because eIF4F complex formation can be increased without a corresponding dissociation of eIF4E from 4E-BPs [19–23], covalent modifications of eIF4E and/or eIF4G might regulate eIF4F assembly [24–27].

There is conflicting evidence about the role of eIF4F in regulating the rate of total protein synthesis in mammalian cells. On the one hand, several mechanisms exist that can decrease the steady-state rate of protein synthesis by suppressing eIF4F activity. Some examples are (1) the sequestration of eIF4E in response to stress or deprivation of nutrients by increasing its binding to 4E-BPs [19,22,28], (2) the cleavage of eIF4G by viral proteases to shut off host protein synthesis [29] and (3) the proteolytic cleavage of eIF4G by caspases during apoptosis [30]. Collectively, these findings would logically suggest that total protein synthesis can be regulated by targeting the activity of eIF4F. On the other hand, increases in total protein synthesis occur without corresponding increases in eIF4F levels [31,32]. Consistent with these findings is the finding that a total blockade of eIF4F complex formation is correlated with a partial decrease in total protein synthesis, whereas proteolytic cleavage of eIF4G in *Xenopus* oocytes has a small effect on total protein synthesis [33–36].

In the present study we hypothesized that an increase in either the quantity of eIF4F complexes or the cap binding affinity of eIF4F by phosphorylation of eIF4E is required for the load-induced acceleration of total protein synthesis in the adult myocardium. The anabolic response to load can be reproduced *in vitro* by electrically stimulated contraction of adult feline cardiocytes [37]. Contractile activity causes tension-dependent increases in eIF4F complex formation and in the extent of eIF4E phosphorylation which are correlated with improved translational efficiency and accelerated rates of total protein synthesis [23,37–39]. The purpose of the present study was to examine how eIF4F complex formation is regulated in the adult cardiocyte and to determine whether modifying eIF4F produces corresponding changes in the rate of total protein synthesis. An adenoviral gene transfer approach was used to express wild-type or mutated forms of eIF4E or the physiological eIF4E kinase Mnk-1. The high efficiency of infection and levels of protein expression make it an effective approach for generating quantitative or qualitative changes in eIF4F complexes in the adult cardiocyte and for measuring the resultant biological effects. The results show the following: (1) eIF4F assembly is increased by raising eIF4E levels via adenoviral gene transfer; (2) the cap binding affinity of eIF4F is a rate-limiting determinant for total protein synthesis rates; and (3) increases in the quantity of eIF4F alone or eIF4E phosphorylation are not sufficient to accelerate total protein synthesis rates.

EXPERIMENTAL

Adult feline cardiocytes in primary culture

Cardiocytes were isolated from the left ventricle of adult feline myocardium as described previously [37]. The cardiocytes were plated in serum-free medium on four-well culture trays (Nunc) coated with laminin; the dimensions of each well were 2.5 cm × 6.5 cm. The initial plating density was 3×10^5 cardiocytes per well. After 4 h of incubation, the cardiocytes were rinsed and maintained in serum-free medium [40]. The cardiocytes were stimulated to contract by delivering 140 V electrical pulses of alternating polarity through the culture medium via carbon electrodes with a custom-built electrical stimulator as described previously [40]. The current between electrodes was approx. 50–55 mA. Cardiocyte contraction was set at a frequency of 2 Hz and a pulse duration of 5 ms. Non-stimulated cardiocytes were quiescent and used as controls.

Adenovirus infection

Replication-defective recombinant adenoviruses were added to cardiocyte suspensions at the time of plating. The infections proceeded over 4 h at a multiplicity of infection of approx. 10 plaque-forming units per cell, as determined by plaque assays. For most experiments the cardiocytes were rinsed in serum-free medium and incubated for 24 h to permit transgene expression. Companion dishes were infected at the same multiplicity of infection with Ad/CMV- β Gal (in which CMV stands for cytomegalovirus), an adenovirus expressing the β -galactosidase gene. The infection efficiency was determined by histochemical staining of the cardiocytes for β -galactosidase activity [41] and by indirect immunofluorescence with an antibody against the influenza haemagglutinin epitope (HA) as described below.

Construction of recombinant adenoviruses

Replication-defective recombinant adenoviruses were generated by homologous recombination with a bacterial system as described by Chartier et al. [42]. The cDNA species for the regions encoding eIF4E and Mnk-1 were cloned into pAD.CMV-Link.1, a shuttle plasmid designed to produce a high level of constitutive expression by replacing E1 adenoviral sequences with a gene cassette consisting of the cytomegalovirus promoter element, the multiple cloning site and the simian virus 40 polyadenylation signal. The cassette was flanked by human adenovirus type 5 sequences consisting of 0–1 map units for the left arm and 9–16 map units for the right arm. To monitor for protein expression, a HA epitope tag was included in the open reading frame on the N-terminus. The mutations in eIF4E were generated by site-directed mutagenesis (Quikchange; Stratagene). All cDNA sequences in the shuttle plasmid were confirmed by nucleic acid sequencing. The purified shuttle plasmid was digested with the restriction enzymes *NheI* and *NarI* or *ApaI* and *NarI* to prepare 'rescue' fragments consisting of the gene cassette and the adenoviral flanking sequences. For homologous recombination, approx. 2 μ g of purified rescue fragment was mixed with 0.1 μ g of the adenoviral plasmid pTG3602 that was linearized with *ClaI*. The plasmid mixture was transformed into competent BJ5183 bacteria for homologous recombination. Bacterial colonies were screened for recombination by restriction enzyme digestion patterns of recombinant adenoviral DNA with *XhoI* and by Southern blotting. Recombinant adenoviral DNA was purified, digested with *PacI* and transfected into the HEK-293 human epithelial kidney cell line by using LIPOFECTAMINE reagent (Gibco BRL).

Figure 1 Infection efficiency of adult feline cardiocytes

(A) Cardiocytes infected with Ad/CMV- β Gal and stained for β -galactosidase activity. (B) Immunofluorescent staining of cardiocytes infected with Ad/HA-eIF4E. Magnification $\times 80$.

Adenoviral plaques were produced approx. 1 week after the transfection of recombinant adenoviral DNA into HEK-293 cells. Plaque lysates were prepared and each recombinant adenovirus was screened for protein expression by Western blotting. The adenoviruses were then plaque-purified [43]. After large-scale propagation, adenoviruses were purified by CsCl gradient centrifugation, dialysed and titred by plaque assay as described [43].

Modifications of eIF4F in cardiocytes

eIF4F complex formation was measured by isolating eIF4E from cell homogenates with γ -m-GTP-Sepharose and measuring the amount of eIF4G bound to eIF4E [17,20,23]. The bound proteins were resolved on SDS/12% (w/v) polyacrylamide gels or 10% (w/v) NUPAGE gels (Novex). In some experiments, aliquots of the unbound fraction were run on the gels. Western blot analyses were performed with either a polyclonal anti-HA antibody (Zymed) or eIF4E and eIF4G antibodies [23]. The attenuances of the corresponding eIF4G and eIF4E bands were measured by digital image analysis. Changes in eIF4F affinity were determined by measuring the extent of eIF4E phosphorylation by one-dimensional isoelectric focusing [38]. The eIF4E isoforms were detected by Western blotting. To quantify the expression of eIF4E, cardiocytes were rinsed in ice-cold PBS and scraped into SDS sample buffer [50 mM Tris/HCl (pH 6.8)/100 mM dithiothreitol/2% (w/v) SDS/10% (v/v) glycerol/0.1% bromophenol blue]. The samples were heated and resolved by SDS/PAGE followed by Western blotting with either eIF4E or anti-HA antibodies. Immunoprecipitation of HA-tagged eIF4E from cell homogenates was done with the polyclonal anti-HA antibody as described previously [23].

Rate of total protein synthesis

The rate of total protein synthesis was measured by pulse labelling as described previously [37]. Cardiocytes were radio-labelled for 4 h with 0.4 mM [3 H]phenylalanine [7 μ Ci/ml], a concentration that facilitates the rapid expansion of the intracellular phenylalanine pool and the equilibration of phenylalanyl-tRNA-specific radioactivity with the specific radio-

activity of phenylalanine in the culture medium. The rate of protein synthesis (nmol of phenylalanine/h per g of protein) was calculated by dividing the specific radioactivity of protein (d.p.m./h per g of protein) by the specific radioactivity of phenylalanine in the culture medium (d.p.m./nmol).

Immunofluorescent detection of HA-tagged eIF4E

Cardiocytes were plated on laminin-coated coverslips and infected with adenovirus. After a 48 h incubation period the cells were fixed in a solution of 4% (w/v) paraformaldehyde in PBS and extracted in PBS containing 0.5% (v/v) Triton X-100. The cells were washed and incubated in blocking solution consisting of 0.1 M glycine and 10% (v/v) donkey serum. The cells were incubated overnight with a murine monoclonal anti-HA antibody (Boehringer Mannheim) at 1:100 dilution. Secondary antibody detection was performed with a HA-FITC donkey anti-mouse antibody (Jackson ImmunoResearch).

RESULTS

Effects of overexpression of eIF4E on eIF4F complex formation

Our previous studies showed that a load-induced increase in eIF4F complex formation was not dependent on the release of eIF4E from 4E-BPs, which suggested that eIF4E could be a limiting component for eIF4F complex formation because of its relatively low abundance or its compartmentation in the adult cardiocyte. To determine the role of eIF4E accessibility in regulating the assembly of eIF4F, cardiocytes were infected with a recombinant adenovirus expressing wild-type HA-tagged eIF4E (HA-eIF4E) on the N-terminus. Figure 1 shows that a multiplicity of infection of 10 was sufficient to infect 91–98% of the cardiocytes as determined by (1) positive histochemical staining for β -galactosidase activity with identical titres of Ad/CMV- β Gal adenovirus and (2) positive staining for HA-eIF4E expression by indirect immunofluorescence with anti-HA antibody.

HA-eIF4E expressed in cardiocytes was distinguished from endogenous eIF4E on Western blots because the HA tag retarded its electrophoretic mobility (Figure 2). The expression of HA-eIF4E in quiescent cardiocytes increased progressively as

Figure 2 Effects of HA-eIF4E overexpression on eIF4F complex formation

(A) Cardiocytes infected with Ad/HA-eIF4E and harvested after 20, 24 and 28 h of incubation. Lanes B, the fraction bound to 3 H-m-GTP-Sepharose (50% of total volume); lanes U, the unbound fraction (10% of total volume). The percentage increases in HA-eIF4E relative to endogenous eIF4E are shown. (B) Repeat experiment in which the percentage increase in HA-eIF4E relative to endogenous eIF4E was smaller at each corresponding time point. (C) Experiment demonstrating effects on eIF4F complex formation after 48 h of incubation. Abbreviations: NI, non-infected; CMV-βGal and HA-eIF4E experiments were performed in duplicate.

measured 20–28 h after infection relative to endogenous eIF4E, thereby expanding the total pool of eIF4E. To measure the effects of HA-eIF4E overexpression on eIF4F complex formation, eIF4E was isolated from cardiocyte homogenates by using 3 H-m-GTP-Sepharose, followed by Western blotting with eIF4G and eIF4E antibodies. Figure 2(A) shows that the amount of eIF4G bound to eIF4E (B) was increased maximally after 20 h, a time point at which HA-eIF4E had increased by 84% relative to endogenous eIF4E. Between 20 and 28 h after infection, HA-eIF4E levels continued to rise without a corresponding increase in the amount of eIF4G bound to eIF4E. The plateau in eIF4F complex formation was not attributable to a limiting amount of eIF4G because a substantial pool of eIF4G remained in the unbound (U) fraction at each time point. These results suggest that accessibility of eIF4E was not the sole determinant regulating eIF4F complex formation.

Figure 2(B) is a repeat experiment in which the percentage increases in HA-eIF4E expression were lower at each corresponding time point, probably because the endogenous eIF4E level in these cardiocytes was greater than in Figure 2(A). These results show that a 66% increase in HA-eIF4E relative to

endogenous eIF4E was sufficient to produce the maximal increase in eIF4G binding. Furthermore, eIF4G binding remained constant in cardiocytes that had extremely high levels of HA-eIF4E overexpression as obtained 48 h after infection (Figure 2C). On the basis of these results, we concluded that the maximal effects on eIF4F complex formation could be achieved with relatively modest increases in HA-eIF4E overexpression as produced 24–28 h after infection.

Several studies have shown that the assembly of eIF4E into eIF4F complexes co-localizes eIF4E with the physiologically relevant kinases. To test further whether HA-eIF4E was incorporated into eIF4F complexes, the phosphorylation state of HA-eIF4E was measured by one-dimensional isoelectric focusing followed by Western blotting (Figure 3A), in which lane 1 shows that the percentage of phosphorylated eIF4E in quiescent cardiocytes infected with CMV-βGal adenovirus was 18%, similar to previous results in non-infected cardiocytes [38]. Overexpression of HA-eIF4E resulted in the appearance of non-phosphorylated and phosphorylated isoforms, although the corresponding isoelectric points were lowered by the presence of the HA epitope tag, as shown previously [44]. The percentage of phosphorylated

Figure 3 Phosphorylation of HA-eIF4E in cardiocytes

(A) eIF4E isoforms resolved by one-dimensional isoelectric focusing. Lane 1, CMV- β Gal-infected; lanes 2, 3 and 4 were infected with HA-eIF4E for 20, 24 and 28 h respectively. Because of its lower isoelectric point, HA-eIF4E obscures the endogenous eIF4E-P band. (B) Cardiocytes infected for 24 h with CMV- β Gal (lanes 1 and 2) or HA-eIF4E (lanes 3 and 4). In the last 4 h, cardiocytes were either treated with 100 nM PMA (lanes 2 and 4) or remained as controls (lanes 1 and 3). (C) Cardiocytes infected for 24 h with CMV- β Gal (lanes 1–3, exposure time 10 s) or HA-eIF4E (lanes 4–6, exposure time 5 s). Lanes 2 and 4, no additional treatment; lanes 3 and 6, electrically stimulated contraction at 2 Hz for 4 h; lanes 1 and 5, co-infection with Ad/HA-Mnk-1.

HA-eIF4E was 16% at 24 h after infection and 18% at 28 h after infection (Figure 3A, lanes 3 and 4). These results indicate that HA-eIF4E exchanged freely with the endogenous eIF4E pool and was incorporated into eIF4F complexes.

To confirm that HA-eIF4E was assembled into functional eIF4F complexes, cardiocytes were treated with the phorbol ester PMA to stimulate eIF4E phosphorylation (Figure 3B). PMA increased endogenous eIF4E phosphorylation from 25% to 37% (Figure 3B, compare lanes 1 and 2) and HA-eIF4E phosphorylation from 15% to 29% (Figure 3B, compare lanes 3 and 4). Thus both eIF4E pools were phosphorylated by PMA, indicating their ability to assemble into eIF4F. Mnk-1 is an eIF4E kinase that binds to eIF4G and has constitutive activity when expressed in cell lines [44]. To establish that HA-eIF4E was a substrate for physiological eIF4E kinases, a HA-tagged Mnk-1 adenovirus was constructed. As shown in Figure 3(C), HA-Mnk-1 increased the phosphorylation of endogenous eIF4E from 5% to 76% in CMV- β Gal-infected cardiocytes (compare lanes 2 and 1). By comparison, electrically stimulated contraction increased eIF4E phosphorylation to 30% (Figure 3C, compare

lanes 3 and 2). Figure 3(C) also shows that the phosphorylation of HA-eIF4E was increased from 13% to 42% after co-infection with HA-Mnk-1 (compare lanes 4 and 5), whereas contraction increased HA-eIF4E phosphorylation to 19%. However, HA-Mnk-1 overexpression did not increase eIF4F complex formation (see Figure 5), which is consistent with recent findings showing that phosphorylation of eIF4E on Ser-209 does not regulate the assembly of eIF4F [44].

The effects of acute anabolic stimuli on eIF4F complex formation were examined (Figure 4). Cardiocytes were infected with Ad/HA-eIF4E and incubated for 24 h to expand the eIF4E pool. In the last 4 h, cardiocytes were treated with insulin or electrically stimulated to contract. In Ad/CMV- β Gal-infected cardiocytes, eIF4F complex formation was increased by either insulin or contraction, which is consistent with previous results in non-infected cardiocytes [23]. Overexpression of HA-eIF4E increased eIF4G binding by an average of 157% (three experiments) in comparison with CMV- β Gal-infected cardiocytes. In HA-eIF4E-infected cardiocytes, the eIF4G-to-eIF4E ratios were respectively $88 \pm 13\%$ and $96 \pm 18\%$ of the untreated control

Figure 4 Effects of eIF4E overexpression on eIF4F complex formation

Cardiocytes were infected for 24 h with either CMV- β Gal (lanes 1–3) or HA-eIF4E (lanes 4–6). During the last 4 h, cardiocytes were either not treated (lanes 1 and 4), treated with 0.1 μ M insulin (lanes 2 and 5) or electrically stimulated to contract at 2 Hz (lanes 3 and 6). eIF4F complex formation was measured by using 50% of the fraction bound to γ m-GTP-Sepharose. Changes in the mobility of eIF4G were probably caused by some degradation of eIF4G on the gel [23]. Summary data of three experiments are given in the text.

value after electrically stimulated contraction and treatment with insulin. Thus, once the pool of eIF4E had reached a concentration sufficient to promote the assembly of eIF4F in the adult cardiocyte, the positive correlation between eIF4F complex formation and an anabolic stimulus was lost. eIF4G was not limiting for eIF4F assembly, as indicated by the presence of eIF4G in the protein fraction that did not bind to γ m-GTP-Sepharose. However, these experiments do not exclude the possibility that a modification of eIF4G is required for assembly.

Figure 5 Effects of HA-Mnk-1 overexpression on eIF4F activity

Cardiocytes were infected for 48 h with either CMV- β Gal (lanes 1–3) or HA-Mnk-1 (lanes 4–6). In the last 4 h, cardiocytes were either not treated (lanes 1 and 4), treated with 0.1 μ M insulin (lanes 2 and 5) or electrically stimulated to contract at 2 Hz (lanes 3 and 6). (A) Phosphorylation of eIF4E isoforms by one-dimensional isoelectric focusing. The expression of HA-Mnk-1 was confirmed by Western blotting with anti-HA antibody as shown. (B) eIF4F complex formation as measured by using 50% of the fraction bound to γ m-GTP-Sepharose.

Table 1 Modifications of eIF4F activity via adenoviral gene transfer

Adenovirus	Mutation	eIF4F complex	eIF4E phosphorylation
HA-eIF4E	Wild-type	Increase	Unaffected
HA-eIF4E/209A	Ser-209 $\rightarrow$ Ala	Increase	Decrease
HA-eIF4E/56F	Trp-56 $\rightarrow$ Phe	Increase	Unaffected
HA-Mnk-1	Wild-type	Unaffected	Increase

Effects of eIF4E phosphorylation on eIF4F complex formation

Another possible mechanism for increasing eIF4F is stabilization of the complex by eIF4E phosphorylation. To determine whether phosphorylation of eIF4E on Ser-209 modifies eIF4F assembly, we infected quiescent cardiocytes with Ad/HA-Mnk-1 and measured its effects on the percentage of phosphorylated eIF4E and on eIF4F complex formation. Figure 5(A) shows that insulin and electrically stimulated contraction increased eIF4E phosphorylation, measured as the percentage of total eIF4E, from 17% to 30% and 36% respectively in CMV- β Gal-infected cardiocytes (compare lane 1 with lanes 2 and 3). As shown in Figure 5(B), there were corresponding increases in eIF4F complex formation. eIF4E phosphorylation increased to 46% in cardiocytes overexpressing HA-Mnk-1, but eIF4F complex formation was not affected (Figure 5B, compare lanes 1 and 4). Overexpression of HA-Mnk-1 enhanced eIF4E phosphorylation to 50% after treatment with insulin and to 60% in response to contraction. However, there were no additive effects on eIF4F complex formation (Figure 5B, compare lanes 2 and 5, and lanes 3 and 6). These results show that (1) HA-Mnk-1 phosphorylated eIF4E that was assembled in the eIF4F complex, (2) eIF4E phosphorylation did not increase eIF4F complex formation, and (3) eIF4E phosphorylation produced in response to anabolic stimuli was potentiated by HA-Mnk-1 overexpression.

Modification of eIF4F activity via adenoviral gene expression

Table 1 summarizes how adenoviral gene transfer was used to modify eIF4F activity. Ad/HA-Mnk-1 was used to increase the phosphorylation of endogenous eIF4E without altering the overall quantity of eIF4F complexes (Figure 5). As shown in Figure 6(A), the mutant HA-eIF4E/209A could not be phosphorylated when expressed at levels comparable to that of wild-type HA-eIF4E. The putative increase in cap binding affinity should therefore not occur on its incorporation into eIF4F. The mutant HA-eIF4E/56F has a decreased affinity for mRNA caps (K_d 1 μ M compared with 0.3 μ M for wild-type eIF4E) [45]. Figure 6(A) shows that HA-eIF4E/56F was phosphorylated to the same extent as wild-type HA-eIF4E. We also compared the ability of HA-eIF4E mutants to assemble into eIF4F complexes in both quiescent and contracting cardiocytes (Figure 6B). HA-tagged eIF4E was immunoprecipitated with anti-HA antibody and the amount of eIF4G bound to eIF4E was determined by Western blotting. In comparison with wild-type HA-eIF4E, similar quantities of eIF4G were recovered in the anti-HA immunoprecipitates derived from cardiocytes infected with either HA-eIF4E/209A or HA-eIF4E/56F.

The assembly of eIF4F complexes was quantified by isolating eIF4E on γ m-GTP-Sepharose and measuring the amount of eIF4G bound to eIF4E (Figure 7A). In quiescent cardiocytes, overexpression of wild-type HA-eIF4E or either HA-eIF4E mutant increased eIF4F complex formation in comparison with

Figure 6 Effects of eIF4E mutations on eIF4F activity

(A) Phosphorylation of eIF4E and HA-eIF4E isoforms by one-dimensional isoelectric focusing. Cardiocytes were infected with the indicated adenoviruses and incubated for 24 h. In the last 4 h, cardiocytes were electrically stimulated to contract at 2 Hz (S) or remained quiescent (Q). (B) Immunoprecipitation of eIF4F complexes with anti-HA antibody. Cardiocytes were infected with adenoviruses for CMV- β Gal (lanes 1 and 5), HA-eIF4E (lanes 2 and 6), HA-eIF4E/56F (lanes 3 and 7) and HA-eIF4E/209A (lanes 4 and 8). After 24 h, cardiocytes were electrically stimulated to contract for 4 h at 2 Hz or remained quiescent as indicated. After isolation of anti-HA immune complexes, Western blotting was performed with eIF4G and eIF4E antibodies.

CMV- β Gal (Figure 7A, compare lanes 2, 3 and 4 with lane 1). In response to electrically stimulated contraction, eIF4F complex formation was increased in CMV- β Gal-infected cardiocytes (Figure 7A, compare lanes 5 and 1). However, there were no additive increases after infection with wild-type HA-eIF4E (Figure 7A, compare lanes 6 and 2), HA-eIF4E/56F (compare lanes 7 and 3) or HA-eIF4E/209A (compare lanes 8 and 4). Figure 7(B) shows summary data calculated by dividing eIF4G by the total amount of eIF4E (endogenous plus HA-tagged). The increase in eIF4F complex formation produced by the overexpression of either wild-type HA-eIF4E or the HA-eIF4E mutants was the same in quiescent and contracting cardiocytes. Because overexpression of HA-eIF4E proteins approximately doubled the total eIF4E pool, the corresponding eIF4G/total eIF4E ratios were decreased by approx. 50% in comparison with the CMV- β Gal-stimulated group. These results further support the conclusion that HA-eIF4E proteins were assembled into eIF4F complexes at comparable levels.

Role of eIF4F activity in regulating the rate of total protein synthesis

To determine how modifying eIF4F affected the rate of total protein synthesis, cardiocytes were infected with the adenoviruses listed in Table 1. Subsequently, the cardiocytes were incubated for 24 h and the rate of total protein synthesis was measured during the final 4 h in the presence or absence of electrically stimulated contraction (Figure 8). Figure 8(A) shows that the wild-type and mutated forms of HA-eIF4E protein were expressed at levels 120–140% above that of endogenous eIF4E and that the levels of overexpression were comparable. Figure 8(B) shows that electrically stimulated contraction significantly accelerated the rate of total protein synthesis in CMV- β Gal-infected cardiocytes in comparison with the quiescent control.

The 27% increase in protein synthesis rate produced after 4 h of contraction was similar to previous findings with non-infected cardiocytes [37,39]. For a direct measure of the effects of increasing the quantity of eIF4F, cardiocytes were infected with Ad/HA-eIF4E to promote the assembly of wild-type eIF4F complexes. The rate of protein synthesis was accelerated by 10% in quiescent cardiocytes; however, this increase was not statistically significant. Furthermore, overexpression of HA-eIF4E had no effect on the ability of contraction to accelerate the rate of total protein synthesis. These results were consistent with the fact that electrically stimulated contraction did not produce an additive increase in the amount of eIF4F complexes. Taken together, these results show that increasing the quantity of eIF4F complexes was not sufficient to accelerate the rate of total protein synthesis.

The role of phosphorylation in regulating the rate of total protein synthesis was determined by using the HA-eIF4E/209A mutant. Although overexpression increased eIF4F complex formation in quiescent cardiocytes (Figure 7), it had no significant effect on the rate of total protein synthesis (Figure 8B). In addition, HA-eIF4E/209A expression did not prevent electrically stimulated contraction from accelerating the rate of total protein synthesis, indicating that eIF4E phosphorylation was not necessary for increasing translational efficiency. The next mutant tested, HA-eIF4E/56F, has a decreased affinity for mRNA caps independently of changes in eIF4E phosphorylation. Overexpression of HA-eIF4E/56F caused an 11% decrease in the rate of total protein synthesis in quiescent cardiocytes and significantly decreased the rate of protein synthesis in electrically stimulated cardiocytes to quiescent values. Because Figures 6 and 7 show that the quantity of eIF4F complexes and the extent of eIF4E phosphorylation were similar to those in cardiocytes infected with wild-type HA-eIF4E, the decrease in the rate of protein synthesis was attributable to the formation of eIF4F

Figure 7 Effects of eIF4E mutations on eIF4F complex formation

(A) Cardiocytes were infected for 24 h with adenoviruses for CMV-βGal (lanes 1 and 5), HA-eIF4E (lanes 2 and 6), HA-eIF4E/56F (lanes 3 and 7) and HA-eIF4E/209A (lanes 4 and 8). In the last 4 h, cardiocytes were electrically stimulated to contract at 2 Hz or remained quiescent as indicated. A Western blot of eIF4F complex formation is shown, as measured by using 50% of the fraction bound to ⁷m-GTP-Sepharose. (B) Summary data of eIF4F complex formation: black bars, quiescent; white bars, stimulated. Values are the means ± S.E.M. ($n = 5$ experiments); * $P < 0.02$ compared with CMV-βGal quiescent group as determined by a paired t test. There were no significant differences between any of the HA-eIF4E-infected groups as determined by an analysis of variance ($n = 4$ experiments).

complexes with decreased cap binding affinity. However, if compared with its quiescent cohort, electrically stimulated contraction accelerated the rate of protein synthesis by 22% in cardiocytes expressing HA-eIF4E/56F ($P < 0.05$, paired t test). These results show that decreasing the affinity of eIF4F decreases the rate of total protein synthesis in quiescent and contracting cardiocytes, which is consistent with the postulated function of eIF4F as a rate-limiting determinant of translational initiation. However, decreasing the cap binding affinity of eIF4F does not impair the ability to accelerate rates of protein synthesis.

It is possible that increasing the formation of eIF4F complexes via the overexpression of eIF4E obviated the role that changes in eIF4E phosphorylation have in accelerating the rate of total protein synthesis. To rule out this possibility, quiescent cardiocytes were infected with Ad/HA-Mnk-1 and then incubated for 48 h to increase endogenous eIF4E phosphorylation without altering the quantity of eIF4F complex (see Figure 5). The rate of total protein synthesis, measured during the final 4 h of incubation, actually decreased from 487 ± 38 nmol of phenylalanine/h per g of protein in CMV-βGal-infected cardiocytes to 417 ± 13 nmol/h per g in HA-Mnk-1-infected cardiocytes (means ± S.E.M. for three experiments). These results show that increasing the eIF4E phosphorylation of existent eIF4F com-

Figure 8 Effects of eIF4E mutations on the rate of total protein synthesis

(A) Cardiocytes were infected with the indicated adenoviruses and incubated for 24 h. In the last 4 h, cardiocytes were electrically stimulated to contract at 2 Hz (S) or remained quiescent (Q). Summary data of six experiments show the expression levels of the eIF4E proteins. Values are means ± S.E.M. A Western blot from one experiment is shown. (B) Rates of total protein synthesis. Values are means ± S.E.M. ($n = 8$ for the CMV-βGal, eIF4E/WT and eIF4E/209A groups, $n = 5$ for the eIF4E/56F group); * $P < 0.05$ compared with the CMV-βGal quiescent group as determined by analysis of variance followed by a Dunnett test.

plexes was not sufficient to accelerate the rate of total protein synthesis in the adult cardiocyte.

DISCUSSION

eIF4F has a constitutive role in regulating a rate-limiting step for translational initiation, namely the binding of mRNA to the 40 S ribosomal subunit. Given this function of eIF4F, it seems logical that rates of total protein synthesis and growth would be directly regulated via changes in eIF4F activity. For example, a positive correlation between eIF4F activity and protein synthesis has been well established in mitotically competent cells [46]. Similarly, the correlation between increases in eIF4F activity and

accelerated rates of total protein synthesis is conserved in terminally differentiated adult cardiocytes, which grow by hypertrophy rather than by cell division [23,47]. Even so, studies have shown that total protein synthesis rates can be accelerated in other types of muscle cells without corresponding changes in either the formation of eIF4F complexes or the extent of eIF4E phosphorylation [31,32,48]. Therefore we used recombinant adenoviruses as a method of high-efficiency gene transfer and determined whether modifications to eIF4F regulate the rate of total protein synthesis at steady state by using quiescent cardiocytes, or in response to increased load by using electrically stimulated contraction.

The results show that the assembly of the eIF4F is controlled at least partly by raising eIF4E levels. Other mechanisms are probably involved because further increases in the eIF4E pool in quiescent cardiocytes did not produce an additive increase in eIF4G binding, despite the fact that a substantial pool of eIF4G was not bound to eIF4E. In contrast with cardiocytes infected with the CMV- β Gal adenovirus, electrically stimulated contraction or insulin did not cause an increase in eIF4F complex formation in cardiocytes overexpressing eIF4E. One possible explanation is that other components of the translational machinery become limiting for assembly once the pool of HA-eIF4E has reached a level that is sufficient to increase eIF4F complex formation. As shown by Khaleghpour et al. [49], an alternative possibility is a feedback loop by which HA-eIF4E triggers the dephosphorylation of 4E-BP1 and conversion to the α -isoform. If this mechanism is operative, its effect would probably be small because it has been shown that a doubling in eIF4E level (a degree of overexpression comparable to adult cardiocytes) produces a small increase in the percentage of the 4E-BP1- α isoform [49]. In these cells, 4E-BP1 consists predominantly of the β and γ isoforms, most of which do not bind to eIF4E. Similarly, we have found that a small percentage of the 4E-BP pool in adult cardiocytes consists of 4E-BP- α [23].

Previous studies have shown that eIF4F complex formation is not necessarily dependent on the release of eIF4E from 4E-BPs [19–23]. The relative percentage of the 4E-BP1 α -isoform is usually low in most cell types, and dephosphorylation of 4E-BP1 becomes pronounced when protein synthesis is suppressed by stress [19,22,28]. In adult cardiocytes, a small percentage of 4E-BP- α indicates that the pathways controlling 4E-BP phosphorylation and its dissociation from eIF4E are apparently active and that eIF4E is not normally sequestered by this particular mechanism. Furthermore, as shown in other cell types, the association between the partly phosphorylated β isoform of 4E-BP1 and eIF4E is apparently weak in adult cardiocytes because the β isoform does not purify together with eIF4E on 3 m-GTP-Sepharose [23]. The interactions between the β isoform and eIF4E are dependent on several mechanisms that regulate the differential phosphorylation of 4E-BP1 on five serine/threonine phosphorylation sites [50]. Consequently, the phosphorylation of 4E-BP1- β on specific sites might generate a pool that is able to sequester eIF4E in the adult cardiocyte. If such a pool exists, it is not large enough to inhibit the increase in eIF4F complexes in cardiocytes overexpressing eIF4E.

Despite the necessity of eIF4F in the process of cap-dependent translation, these results revealed differential effects on eIF4F activity and total protein synthesis rates. Overexpression of eIF4E increases the quantity of eIF4F complexes but it is not sufficient to accelerate the rate of total protein synthesis or to potentiate the anabolic effects of increased load as produced by contraction. We cannot exclude the possibility that eIF4F complex formation is necessary for accelerating protein synthesis rates because overexpression of either wild-type or mutated

eIF4E increased the quantity of eIF4F complexes in quiescent and contracting cardiocytes. Furthermore, the results show that the cap binding affinity conferred by eIF4E on the eIF4F complex is sufficient, and that it is not necessary to increase the extent of eIF4E phosphorylation on Ser-209 to accelerate the rate of total protein synthesis. These conclusions are supported by our findings that the percentage of phosphorylated eIF4E is normally low in the adult cardiocyte [38] and that it remains low even when eIF4E is overexpressed to increase eIF4F assembly.

The results show that eIF4E phosphorylation does not stabilize the eIF4F complex or regulate the rate of total protein synthesis. It is possible that the assembly of eIF4E into the eIF4F complex causes an affinity change that enhances cap binding, as shown *in vitro* [14]. We speculate that the resultant cap binding affinity of the eIF4F complex is sufficient to initiate the translation of most mRNA species. When we lowered the affinity for mRNA caps by overexpression of HA-eIF4E/56F, the rate of total protein synthesis was decreased. However, total protein synthesis was accelerated by contraction in cardiocytes infected with HA-eIF4E/56F, even though the absolute value remained significantly lower than in cardiocytes overexpressing wild-type eIF4E. We conclude that the affinity of eIF4F for the cap provides a set point for overall initiation rates but that decreasing the affinity of eIF4E does not impair the ability to accelerate the rate of total protein synthesis. Because eIF4F complex formation was increased in cardiocytes expressing HA-eIF4E/56F, it could have compensated for the decrement in cap binding affinity of this mutant.

It is well established that myocardial protein synthesis rates are accelerated during load-induced hypertrophic growth [4]. Translational mechanisms are involved because mRNA species must compete for a limited quantity of ribosomes to initiate translation. An increase in either eIF4F complexes or eIF4E phosphorylation is not necessary or sufficient to accelerate total protein synthesis rates. Most constitutively expressed mRNA species probably have 5'-untranslated regions that are conducive to high efficiencies of translation and therefore do not require modifying eIF4F to increase ribosome binding. However, increasing eIF4F activity could function by increasing ribosome binding to a small subset of mRNA species that have highly structured 5'-untranslated regions and lower efficiencies of translation [46]. Many of the mRNA species in this subset encode growth-related proteins whose synthesis is tightly controlled and ordinarily low at steady state. Furthermore, as the ribosome pool expands during hypertrophy, a corresponding increase in eIF4F complexes would be necessary to maintain overall initiation rates and thereby sustain the efficiency of total protein synthesis at high levels.

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