Biochemical and Biophysical Research Communications 426 (2012) 129–134

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

RGS2 is a component of the cellular stress response

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ARTICLE INFO

Article history:
Received 31 July 2012
Available online 17 August 2012

Keywords:
RGS protein
Translation
Protein synthesis
Cellular stress response

ABSTRACT

Regulator of G protein signaling (RGS) proteins are GTPase accelerating proteins for heterotrimeric G protein α-subunits. RGS2 has recently been shown to have additional G protein-independent functions including control of ion channel currents, microtubule polymerization, and protein synthesis. Cellular levels of RGS2 mRNA and protein are upregulated in response to various forms of stress suggesting that it may be a stress-adaptive protein; however, direct evidence to support this notion has remained elusive. In this report, we show that thermal stress upregulates RGS2 expression and this serves to arrest *de novo* protein synthesis. The latter is an established cellular response to stress. Inhibiting the stress-induced RGS2 upregulation by way of siRNA knockdown diminished the repression of global protein synthesis. The collective results of our study implicate RGS2 upregulation as a cellular mechanism of controlling *de novo* protein synthesis in response to stress. This work provides greater insight into the stress proteome and the role of RGS2.

1. Introduction

Cells are constantly being subjected to stressful conditions throughout their lifetime. This can include exposure to such factors as nutrient deprivation, hypoxia, oxidative damage, DNA damage, viral infection, and temperature fluctuations. In response to these harmful stimuli cells will activate both pro- and anti-apoptotic pathways to ensure that neither aberrant cellular survival nor erroneous cellular death occurs. In the case of the latter, cells have evolved a collection of complex mechanisms to recover from these insults known as the cellular stress response (CSR) [1,2]. Ultimately, the CSR serves to coordinate gene expression at the levels of DNA transcription and protein translation.

A key feature of the CSR is a global reduction in protein synthesis coupled with an increase in the expression of specific stress-associated proteins [2]. This paradoxical control of protein biogenesis serves to prevent the unnecessary expenditure of energy while providing a means to combat the immediate threat, and is typically achieved by phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α) by four distinct stress-activated kinases [3]. Phosphorylation at the conserved serine residue converts eIF2 from a substrate into a competitive inhibitor of its guanine nucleotide exchange factor, eIF2B, and impedes the rate-limiting step of translation initiation [4]. However, through alternative translation mechanisms using, for example, upstream open reading frames and internal ribosome entry sites, cells are able to upregulate specific components of their stress proteome such as transcription factors [5]. By elevating the levels and activity of transcription factors, cells can in turn increase the transcript of additional genes needed for maintaining homeostasis.

Although phosphorylation of eIF2α is a hallmark component of the CSR, the observation that protein synthesis remains moderately inhibited in a transgenic mouse embryonic fibroblast (MEF) cell line expressing a nonphosphorylatable form of eIF2α ($eIF2^{A/A}$) [6,7] suggests that additional mechanisms exist for controlling the translational machinery in response to stress. Recent work from our lab characterized a previously unrecognized aspect of translational control that involves RGS2 (regulator of G protein signaling 2) [8]. RGS2 is a member of the regulator of G protein signaling proteins that serve as GTPase accelerating proteins (GAPs) for heterotrimeric G proteins [9]. Additional G protein-independent functions have also been established for RGS2 [10–12]. The earlier observations that RGS2 mRNA and protein expression can be upregulated by various forms of stress suggested that it may be a stress-adaptive protein [13–16]; however, direct evidence to support this notion remains lacking. This report provides data in support of the role for RGS2 in the CSR. We show that stress-dependent RGS2 upregulation is governed independently of eIF2α phosphorylation, and this increase in RGS2 expression serves to complement translational control by eIF2. The current study contributes to the growing body of literature supporting eIF2α phosphorylation-independent mechanisms of gene regulation.

Abbreviations: ATF, activating transcription factor; CSR, cellular stress response; eIF, eukaryotic initiation factor; GAP, GTPase accelerating protein; MEF, mouse embryonic fibroblast; RGS, regulator of G protein signaling; UPR, unfolded protein response; XBP, X-box binding protein.

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http://dx.doi.org/10.1016/j.bbrc.2012.08.050

2. Materials and methods

2.1. Reagents

eIF2α phospho-S51 antibody was purchased from Cell Signaling Technology, Inc. (Boston, MA). [^{3}H]-leucine was purchased from Perkin Elmer (Waltham, MA). RGS2 Mission siRNA and universal negative control were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON). Most other reagents were purchased from Invitrogen Canada Inc. (Burlington, ON).

2.2. Cell culture

Mouse embryonic fibroblast eIF2$^{S/S}$ and eIF2$^{A/A}$ cells [6] were a gift from Dr. Randal Kaufman (Sanford-Burnham Medical Research Institute, La Jolla, CA) and maintained as described [8].

2.3. Viability assay

MEF cells were grown in 96-well cluster plates and then subjected to heat shock by incubation in a 45 °C water bath for 30 min. Cells were then allowed to recover in the culture incubator at 37 °C for 4 h or not at all. The MTS assay (Promega Corp., Madison, WI) was then performed according to the manufacturer's protocol.

2.4. RNA sample preparation

Cells in 6-well cluster plates were washed twice with ice-cold PBS then lysed with Trizol reagent. Samples were transferred to microcentrifuge tubes and 1 part chloroform was added to 5 parts Trizol reagent. The mixture was mixed by vigorous shaking followed by incubation at room temperature for 2 min. Samples were then centrifuged at 10,000×*g* for 15 min at 4 °C. The aqueous phase containing RNA was transferred to new microcentrifuge tubes and an equal volume of isopropanol was added. Samples were mixed by gentle inversion six times, incubated at room temperature for 10 min, and the precipitated RNA was pelleted by centrifugation at 10,000×*g* for 10 min at 4 °C. Supernatants were discarded and the resulting pellet was washed with 75% ethanol. Pellets were then allowed to air dry for approximately 10 min and then resuspended in diethyl pyrocarbonate (DEPC)-treated water. RNA samples were quantified and checked for purity using UV spectrophotometry.

2.5. Real-time PCR

First strand cDNA synthesis from purified RNA was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems Canada, Streetsville, ON). The newly synthesized cDNA was subjected to real-time PCR on an ABI 7900HT Fast System (Applied Biosystems Canada, Streetsville, ON) using Taqman Universal PCR master mix, FAM-labeled Taqman Gene Expression assay for RGS2 (Mm00501385), and VIC-labeled Taqman Gene Expression assay for mouse GAPDH (4352339E) (Applied Biosystems Canada, Streetsville, ON). Relative mRNA levels were quantified using the $\Delta\Delta C_t$ analysis method using GAPDH as internal control (http://www.pathmicro.med.sc.edu/pcr/pcr-home.htm). All operations were performed according to the manufacturers' recommended protocols.

2.6. Protein synthesis assay

Cells grown in 6-well cluster plates were subjected to heat shock by incubation in a 45 °C water bath for 30 min. The medium was then replaced with identical culture medium supplemented with 0.5 μCi/ml [^{3}H]-leucine and allowed to recover at 37 °C for the indicated times. *De novo* protein synthesis was determined as described [8].

2.7. siRNA knockdown

Cells were seeded in 6-well cluster plates and grown to a confluency of approximately 50% prior to transfection with the indicated siRNA (100 pmol/well) using lipofectamine 2000 reagent (Invitrogen Canada Inc., Burlington, ON) according to the manufacturer's protocol.

2.8. Immunoblot analysis

Proteins were visualized as described [8].

3. Results

3.1. Heat shock of eIF2$^{S/S}$ and eIF2$^{A/A}$ mouse embryonic fibroblasts does not induce cell death

The generation of eIF2$^{A/A}$ MEF cells [6] resulted in an invaluable tool for studying the CSR, as it permits for dissecting the eIF2α phosphorylation-dependent and independent pathways. Consistent with previous works, immunoblot analysis of eIF2$^{A/A}$ MEF cell lysates determined no immunoreactive band arising from heat shock treatment using an eIF2α-Ⓟ S51 antibody (Fig. 1A). This contrasts with what is observed in parallel assays with control wild-type cells that respond with robust eIF2α phosphorylation which peaks at approximately 15 min and returns to basal levels within 1 h (Fig. 1A). We also examined the viability of both cell lines following heat shock to rule out the possibility that eIF2$^{A/A}$ cells were not exhibiting eIF2α phosphorylation as a result of enhanced cell death. Viability was inferred by comparing the mitochondrial metabolic activity of cells immediately after heat shock treatment or following a 4 h recovery period. There were no differences in cell viability noted between the two cell lines or in response to heat shock treatment at the time points examined (Fig. 1B and C). These results indicate that heat shock conditions used in the current study do not induce cell death in eIF2$^{S/S}$ or eIF2$^{A/A}$ MEF cells.

3.2. RGS2 mRNA is differentially upregulated in eIF2$^{S/S}$ and eIF2$^{A/A}$ MEF cells

We next looked at the effect of heat shock on acute RGS2 mRNA expression, and surprisingly, we observed a difference in the RGS2 expression pattern between eIF2$^{S/S}$ and eIF2$^{A/A}$ cells. RGS2 mRNA levels did not increase to any appreciable level in eIF2$^{S/S}$ cells until 3 h following heat shock whereas the levels of RGS2 in eIF2$^{A/A}$ cells was clearly increased at 90 min post-stress and continued to rise for the duration of the time points examined (Fig. 2). Thus, the data clearly show that RGS2 mRNA is upregulated in MEF cells following heat shock, and this increase in RGS2 expression is more rapid and robust in eIF2$^{A/A}$ cells. These results suggest that RGS2 upregulation may be a complementary and/or compensatory stress response mechanism to eIF2α phosphorylation.

3.3. eIF2$^{A/A}$ cells exhibit a latent heat shock-mediated inhibition of protein synthesis

To further investigate how the lack of eIF2α phosphorylation affects the cellular stress response, we examined the effect of heat shock on *de novo* protein synthesis over a time frame that would

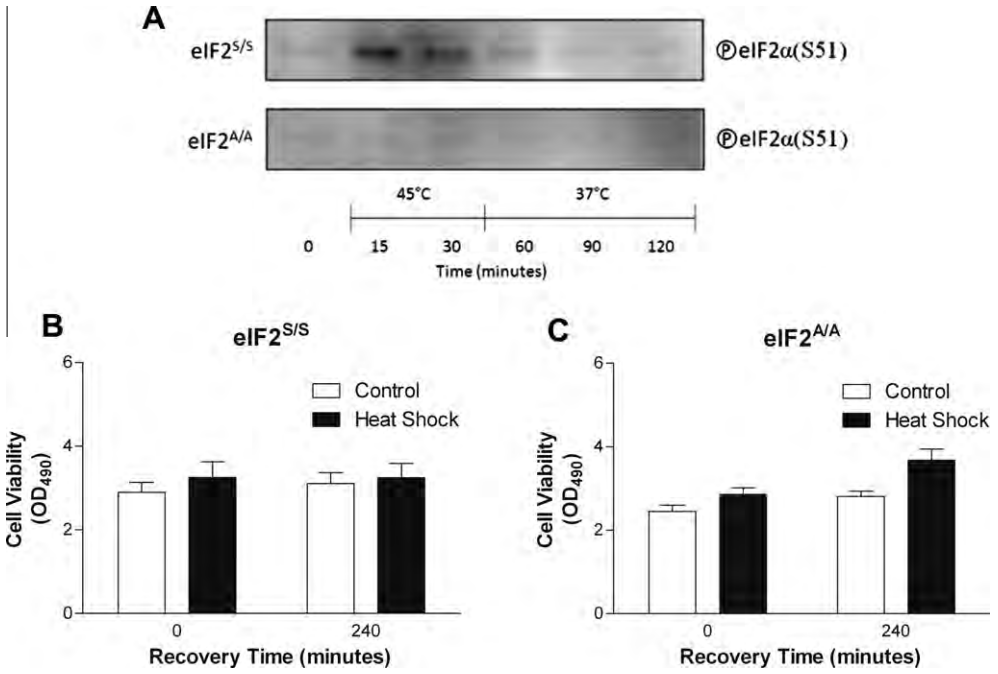

Fig. 1. Heat shock induces phosphorylation of eIF2α at serine 51 but does not promote cell death. (A) MEF cells were incubated at 45 °C for 30 min and allowed to recover at 37 °C for the indicated times. Total protein from cell lysates (30 μg) were resolved on an SDS gel, transferred to PVDF membrane, and the membranes were subsequently probed with anti-ⓅeIF2α(S51) antibody and visualized by chemiluminescence. The blots shown are representative of 3 independent experiments. (B and C) MEF cells were incubated at 45 °C for 30 min and then examined for cell viability immediately or after a 4 h recovery period at 37 °C as detailed in Section 2. The data are presented as the mean ± S.E.M. of three independent experiments.

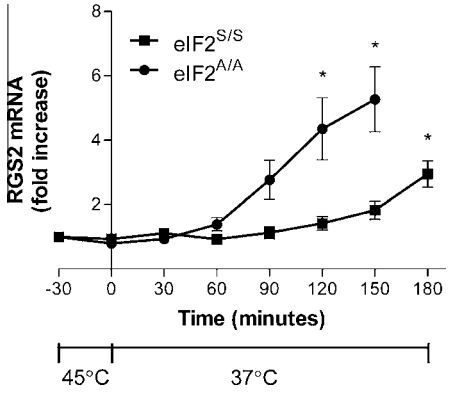

Fig. 2. RGS2 mRNA is differentially upregulated in MEF cells in response to heat shock. eIF2$^{S/S}$ and eIF2$^{A/A}$ cells were incubated at 45 °C for 30 min and then allowed to recover at 37 °C for the indicated times. Total RNA was purified using Trizol reagent and RGS2 mRNA was quantified using real-time RT-PCR. The data have been normalized to basal RGS2 mRNA levels and are presented as the mean ± S.E.M. of four independent experiments performed in duplicate. $^{*}p < 0.05$ versus matched basal control (One-way ANOVA).

take into consideration any temporal aspects of translational control. In control cells heat shock resulted in an immediate and robust inhibition of protein synthesis that was observed as early as 30 min and continued throughout the 4 h time-course of the experiment (Fig. 3A). This rapid onset for the attenuation of protein production coincides nicely with the phosphorylation of eIF2α that was previously noted to peak in eIF2$^{S/S}$ cells at approximately 15 min post-heat shock (Fig. 1A). However, when eIF2α phosphorylation was not a factor there was no appreciable decrease in protein synthesis initially, but to our surprise, a significant reduction in protein synthesis became evident approximately 2–3 h after heat shock (Fig. 3B). The lack of an initial decrease in protein synthesis (e.g. ⩽2 h) in eIF2$^{A/A}$ cells is consistent with the absence of stress-dependent eIF2α phosphorylation in these cells; however, the protein synthesis profile at the later time points (⩾2 h) suggests that an alternative and delayed mechanism of translational control is activated independently of eIF2α phosphorylation.

3.4. siRNA knockdown of RGS2 attenuates the latent inhibition of protein translation

The coinciding time frames of RGS2 mRNA induction and the curtailing of *de novo* protein synthesis following heat shock in the eIF2$^{A/A}$ cells suggested to us that RGS2 may be involved with the delayed protein synthesis regulation. To further investigate this possibility we used an siRNA knockdown approach to examine whether heat shock-dependent RGS2 upregulation was necessary for the observed effects on protein production. Under the conditions used for these studies we were able to obtain approximately 40% and 60% knockdown of RGS2 mRNA in eIF2$^{S/S}$ and eIF2$^{A/A}$ MEF cells, respectively (Fig. 4A and B). We found that by knocking down the levels of RGS2 transcript, the delayed inhibition of protein synthesis observed in eIF2$^{A/A}$ cells following heat shock was decreased (Fig. 4D). In contrast, RGS2 knockdown caused no observable change with regard to the effects of temperature on global protein synthesis in eIF2$^{S/S}$ cells, as these cells exhibited similar heat shock-induced decreases with RGS2 siRNA treatment as with control siRNA (Fig. 4C). These results suggest that RGS2 is an important factor in the latent and eIF2α phosphorylation-independent inhibition of protein synthesis following stress.

4. Discussion

Cells respond to harmful stimuli by activating the CSR and upregulating their stress proteome. This permits for cell recovery and helps to avoid apoptosis. An important component of the CSR is the global reduction of protein synthesis (also known as translation), which is divided into the stages of initiation, elongation, and

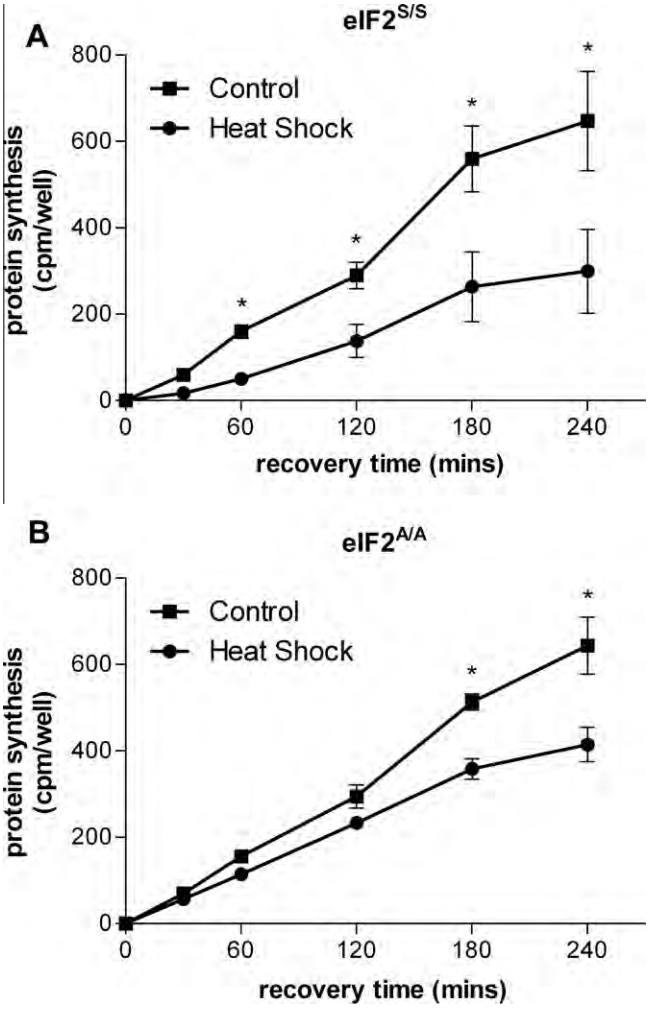

Fig. 3. Heat shock-mediated inhibition of protein synthesis is delayed in eIF2$^{A/A}$ MEF cells. (A) eIF2$^{S/S}$ and (B) eIF2$^{A/A}$ cells were incubated at 45 °C for 30 min. The medium was then replaced with identical growth medium but containing 0.5 μCi/ml [^{3}H]-leucine and cells were allowed to recover at 37 °C for the indicated times. Cellular lysates were collected and [^{3}H]-leucine incorporation into newly synthesized protein was measured as described in Section 2. The data are presented as the mean ± S.E.M. of three independent experiments performed in duplicate. $^*p < 0.05$ versus matched time control (Two-way ANOVA).

termination [17]. Translation is governed principally at the level of initiation as it is more efficient to control its onset than it is to interrupt the process. Thus, the activities of a number of initiation factors (eIFs) have been identified as checkpoints for translational control during stress – the two most prominently studied being eIF2 and eIF4 [2]. As noted previously, phosphorylation of eIF2α on serine 51 significantly impedes protein synthesis by interfering with the eIF2–eIF2B guanine nucleotide exchange cycle and the subsequent loading of initiator methionine onto the small ribosomal subunit [4]. Dephosphorylation of eIF2α by GADD34 and CReP then ensues through the activation of a negative feedback loop, which typically occurs within an hour depending on the type of insult and biological system employed [18–21]. Yet interestingly, the repression of global protein synthesis can be sustained for several hours (Fig. 3) following eIF2α dephosphorylation indicating that other translation control mechanisms are likely involved. The current work, in conjunction with our earlier characterization of RGS2 interactions with eIF2Bε [8], suggests the induction of RGS2 expression may serve as a previously unrecognized mechanism by which global protein synthesis is decreased in response to stress. This putative role for RGS2 in the CSR would be consistent with its previous characterization as an immediate early response gene [14,22,23].

The present study suggests that translational control by RGS2 occurs independently of eIF2α phosphorylation but may serve to temporally complement the latter. Protein synthesis is significantly decreased in eIF2$^{A/A}$ cells following heat shock, although this response is delayed in comparison to wild-type cells, and this effect can be reduced by knocking down RGS2 mRNA expression using an siRNA approach (Fig. 4). Thus, it is conceivable that RGS2 upregulation may act as a secondary stress response mechanism for controlling protein synthesis to complement the immediate effects of eIF2α phosphorylation. This role for RGS2 in the CSR adds to the growing list of studies that have identified eIF2α phosphorylation-independent mechanisms of stress-dependent gene regulation, which include the eIF4E-binding proteins that interfere with mRNA recruitment to the ribosome [24] as well as cytoplasmic stress granules that are associated with mRNA degradation [25].

The CSR is actually composed of a number of different but overlapping divisions including the heat shock response, oxidative stress response, and the unfolded protein response pathways. As its name implies, the UPR represents a means for cells to detect protein misfolding through the use of three key "sensors" within the endoplasmic reticulum membrane which ultimately signal to transcription factors [26]. These include PERK (double-stranded RNA-dependent protein kinase-like ER kinase), IRE1α (inositol-requiring 1α), and ATF6. The PERK pathway involves the phosphorylation of eIF2α and the selective upregulation of the transcription factor ATF4 protein expression through an alternative translation mechanism involving ribosome shunting and two upstream open reading frames [6,27]. In this way, elevations in ATF4 protein levels further drive the expression of other stress-associated genes. The observation that RGS2 transcript levels are increased more so and faster in eIF2$^{A/A}$ than eIF2$^{S/S}$ cells suggests that transcriptional control of RGS2 in response to heat shock may not be principally regulated by ATF4. The transcription factor(s) that is responsible for upregulating RGS2 expression has yet to be identified. One might presume the most likely candidate would be the family of heat shock transcription factors given the stress used in the present study is heat shock itself; however, the promoter region of RGS2 does not contain any binding sites for established heat shock response factors [14]. This is surprising given that the RGS2 promoter region is relatively large in comparison to its coding sequence and hosts a number of binding sites for transcription factors that are implicated in a range of biological processes. Another likely candidate would be the Sp1 (specificity protein 1) transcription factor. Work looking into the role of RGS2 in adipocyte differentiation determined that one of two Sp1 binding sites (Sp1-A) within the RGS2 promoter region was necessary for 3T3-L1 cell differentiation, and this response occurred in a cAMP-dependent manner [28]. It is likely that transcriptional control of RGS2 is multifaceted and dependent upon both the stimulus and cellular context, which would be consistent with the fact that RGS2 expression can be induced by an array of factors in a number of different cell types [13–16,23,28–32].

Although eIF2α phosphorylation does not appear to have a direct role in RGS2 transcriptional control, this does not preclude the possibility that it may be involved in translational control of RGS2 protein expression. As mentioned above, the overall rate of protein synthesis in response to stress is decreased but a subset of mRNAs can be selectively translated through alternative translation mechanisms. We and others have previously shown evidence that the induced expression of RGS2 protein tends to yield multiple bands of similar size in immunoblot analyses [8,33]. It was unclear whether the additional bands were other RGS2 species, post-translationally

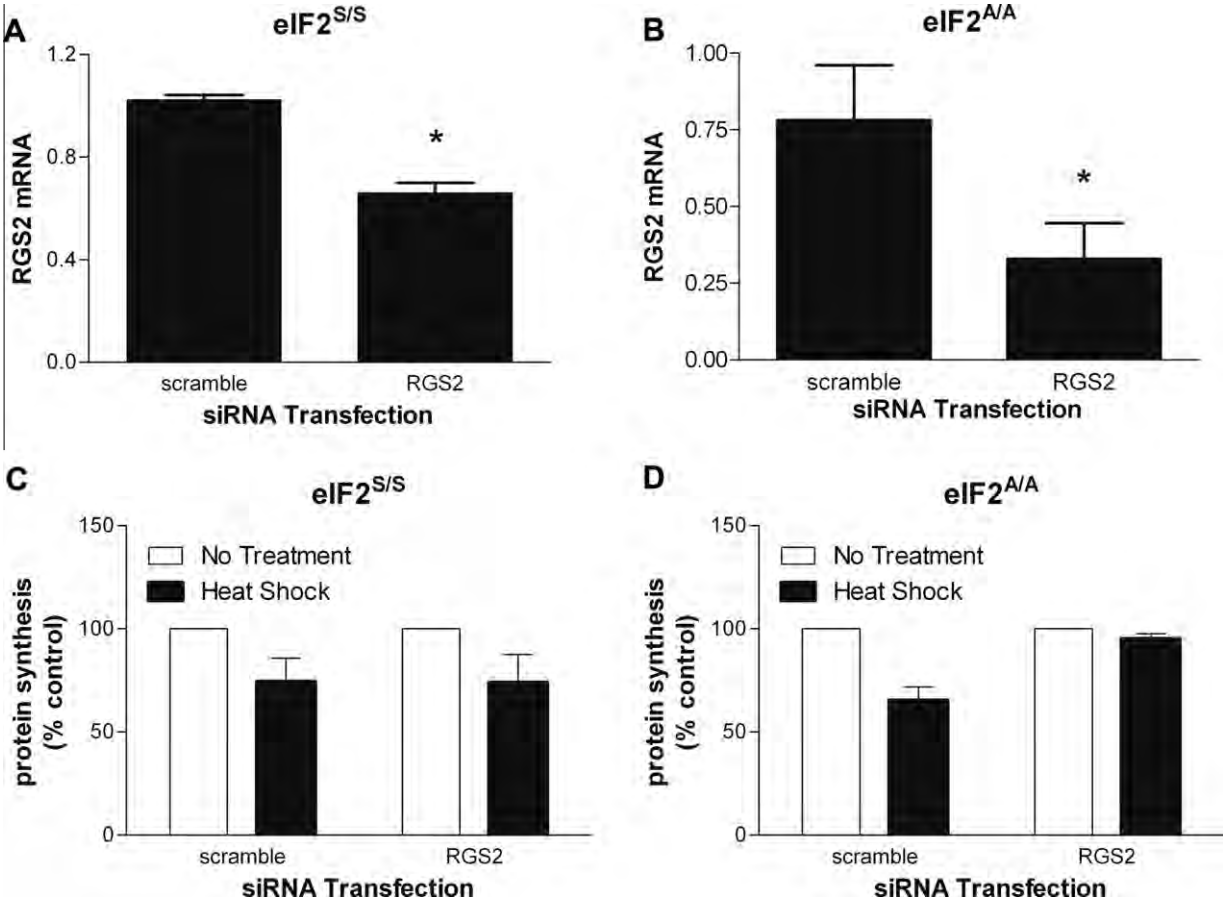

Fig. 4. siRNA knockdown of RGS2 attenuates the delayed stress-dependent inhibition of protein synthesis. (A & B) MEF cells were transfected with RGS2 siRNA or a universal negative control and subsequently analyzed for RGS2 mRNA expression as described in Section 2. $^{*}p < 0.05$ versus control (paired *t*-test). (C and D) MEF cells were transfected with the indicated siRNA. At 48 h post-transfection cells were incubated at 45 °C for 30 min. The medium was then replaced with identical growth medium but containing 0.5 μCi/ml [^{3}H]-leucine and cells were allowed to recover at 37 °C for 4 h. Cellular lysates were collected and [^{3}H]-leucine incorporation into newly synthesized protein was measured as described in Section 2. The data have been normalized to non-heat shock controls for each transfection condition and are presented as the mean ± S.E.M. of three independent experiments. Raw values for non-heat shock controls (mean ± S.E.M.): S/S-scramble, 1033 ± 346; S/S-RGS2, 1096 ± 373; A/A-scramble, 1650 ± 764; A/A-RGS2, 1156 ± 613.

modified RGS2, or were simply artifacts due to the limitations of the currently available antibodies for detecting endogenous RGS2. This dilemma was recently resolved by a study that identified alternative translation initiation of RGS2 as a means for generating multiple protein species [34]. The four protein products resulting from translation initiation at residues 1, 5, 16, and 33 had similar properties with respect to inhibition of $G\alpha_q$ signals, but RGS2 arising from residues 16 and 33 had impaired inhibition of adenylyl cyclase type V activity. The possibility that alternative translation initiation of RGS2 might serve as a stress response mechanism is very intriguing and would be in line with what is observed for other stress-induced proteins such as ATF4. However, additional studies are warranted before further conclusions can be drawn.

Acknowledgments

We are grateful to Dr. Randal Kaufman (Sanford-Burnham Medical Research Institute, La Jolla, CA) who provided us with the mouse embryonic fibroblast cell lines which were an invaluable research tool for our studies. This work was supported by operating grants from the Canadian Institutes of Health Research and the Heart and Stroke Foundation of Ontario. P.C. holds a Career Investigator Award from the Heart and Stroke Foundation of Ontario.

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