Published in final edited form as:
J Cell Physiol. 2013 July ; 228(7): . doi:10.1002/jcp.24327.

TGFβ-Induced PI 3 Kinase-Dependent Mnk-1 Activation is Necessary for Ser-209 Phosphorylation of eIF4E and Mesangial Cell Hypertrophy

FALGUNI DAS[1], **NANDINI GHOSH-CHOUDHURY**[2,3], **AMIT BERA**[1], **BALAKUNTALAM S. KASINATH**[1,3], and **GOUTAM GHOSH CHOUDHURY**[1,3,4,*]

[1]Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, Texas

[2]Department of Pathology, University of Texas Health Science Center at San Antonio, San Antonio, Texas

[3]VA Research, South Texas Veterans Health Care System, San Antonio, Texas

[4]Geriatric Research, Education and Clinical Center, South Texas Veterans Health Care System, San Antonio, Texas

Abstract

Transforming growth factorβ (TGFβ)-induced canonical signal transduction is involved in glomerular mesangial cell hypertrophy; however, the role played by the noncanonical TGFβ signaling remains largely unexplored. TGFβ time-dependently stimulated eIF4E phosphorylation at Ser-209 concomitant with enhanced phosphorylation of Erk1/2 (extracellular signal regulated kinase1/2) and MEK (mitogen-activated and extracellular signal-regulated kinase kinase) in mesangial cells. Inhibition of Erk1/2 by MEK inhibitor or by expression of dominant negative Erk2 blocked eIF4E phosphorylation, resulting in attenuation of TGFβ-induced protein synthesis and mesangial cell hypertrophy. Expression of constitutively active (CA) MEK was sufficient to induce protein synthesis and hypertrophy similar to those induced by TGFβ. Pharmacological or dominant negative inhibition of phosphatidylinositol (PI) 3 kinase decreased MEK/Erk1/2 phosphorylation leading to suppression of eIF4E phosphorylation. Inducible phosphorylation of eIF4E at Ser-209 is mediated by Mnk-1 (mitogen-activated protein kinase signal-integrating kinase-1). Both PI 3 kinase and Erk1/2 promoted phosphorylation of Mnk-1 in response to TGFβ. Dominant negative Mnk-1 significantly inhibited TGFβ-stimulated protein synthesis and hypertrophy. Interestingly, inhibition of mTORC1 activity, which blocks dissociation of eIF4E-4EBP-1 complex, decreased TGFβ-stimulated phosphorylation of eIF4E without any effect on Mnk-1 phosphorylation. Furthermore, mutant eIF4E S209D, which mimics phosphorylated eIF4E, promoted protein synthesis and hypertrophy similar to TGFβ. These results were confirmed using phosphorylation deficient mutant of eIF4E. Together our results highlight a significant role of dissociation of 4EBP-1-eIF4E complex for Mnk-1-mediated phosphorylation of eIF4E. Moreover, we conclude that TGFβ-induced noncanonical signaling circuit involving PI 3 kinase-dependent Mnk-1-mediated phosphorylation of eIF4E at Ser-209 is required to facilitate mesangial cell hypertrophy.

*Correspondence to: Goutam Ghosh Choudhury, Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, Texas. choudhuryg@uthscsa.edu.

The authors do not have any conflict of interest.

Additional supporting information may be found in the online version of this article.

Progressive renal disease is characterized by mesangial expansion. Mesangial cells comprise approximately 2% of kidney cells and represent an integral component of glomerular capillaries (Abboud, 2012). During glomerular disease process a myriad of soluble growth factors and cytokines including transforming growth factorβ (TGFβ) are secreted by infiltrating macrophages and resident kidney cells (Eddy and Neilson, 2006). Transgenic mice overexpressing TGFβ exhibit nephropathy (Kopp et al., 1996). Glomerular upregulation of TGFβ has been demonstrated in both experimental and human kidney disease (Iwano et al., 1996; Bottinger, 2007). Moreover, neutralization of TGFβ ameliorates renal complications of diabetes (Ziyadeh et al., 2000). Glomerular mesangial cells undergo hypertrophy and produce matrix proteins in response to TGFβ. Accumulation of matrix proteins in the mesangium encroaches on and decreases the surface area available for glomerular filtration, leading to progressive mesangial expansion, glomerulosclerosis and end stage renal disease (Abboud, 2012).

TGFβ binds to its high affinity type II receptor, which forms an oligomeric complex with the type I receptor; the latter is constitutively bound to the FK506 binding protein FKBP12. Upon ligand binding type II receptor phosphorylates type I receptor at the GS domain to release FKBP12; this opens the L45 loop permitting interaction with the L3 loop of the receptor-specific Smad 3 and Smad 2 (Shi and Massague, 2003). The FYVE domain containing protein SARA facilitates the binding of Smad to the receptor (Tsukazaki et al., 1998). Activated type I receptor then phosphorylates the C-terminal SVXX motif of Smads 2 and 3, resulting in their release from the receptor complex (Shi and Massague, 2003). Phosphorylated Smads heterodimerize with common Smad, Smad 4, and translocate to the nucleus to recruit transcriptional coactivators or corepressors to regulate target gene transcription. We have shown that TGFβ-stimulated noncanonical phosphatidylinositol (PI) 3 kinase/Akt signaling contributes to the canonical Smad 3-dependent gene transcription in mesangial cells, indicating cooperation of between the two pathways (Das et al., 2008b). Furthermore, we have demonstrated a role of mechanistic target of rapamycin (mTOR) in regulating proteins that take part in initiation of mRNA translation necessary for mesangial cell hypertrophy (Das et al., 2008a).

4EBPs bind to eIF4E and negatively regulate the formation of eIF4F complex. Phosphorylation of 4EBP-1 by mTORC1 induces its dissociation from eIF4E (Sonenberg and Hinnebusch, 2007). Free eIF4E then binds to the scaffolding protein eIF4G and RNA helicase eIF4A to form eIF4F complex. Mitogenic and stress signaling activates phosphorylation of eIF4E by Mnk serine/threonine kinase at Ser-209 (Waskiewicz et al., 1999). Two predominant isoforms of mitogen-activated protein kinase signal-integrating kinase-1 (Mnk-1) and Mnk-2 are ubiquitously expressed. Mnk-1/2 double knock out mice show lack of phosphorylation of eIF4E in all organs studied, suggesting that these kinases represent the only eIF4E kinase in mammals (Ueda et al., 2004). Although extracellular signal regulated kinase1/2 (Erk1/2) signaling regulates phosphorylation of eIF4E, certain cytokines and stresses use p38 MAPK to induce its phosphorylation (Morley and McKendrick, 1997). We have recently shown that TGFβ-stimulated mTORC1 phosphorylates 4EBP-1 resulting in its dissociation from eIF4E (Das et al., 2008a). No information is available on the regulation of phosphorylation of eIF4E by TGFβ. Furthermore, the role of eIF4E phosphorylation at Ser-209 in TGFβ-induced mesangial cell hypertrophy has not been investigated. In the present study, we investigated the role of TGFβ-induced eIF4E Ser-209 phosphorylation in mesangial cell hypertrophy. We find that TGFβ activates Mnk-1 via a signaling cascade involving PI 3 kinase and Erk1/2 to increase phosphorylation of eIF4E. We show that dissociation of eIF4E from 4EBP-1 is necessary for its phosphorylation at Ser-209 and an essential step for TGFβ-induced mesangial cell hypertrophy.

Materials and Methods

Reagents

Tissue culture materials and fetal bovine serum were purchased from Invitrogen (Grand Island, NY). Recombinant TGFβ was purchased from R & D Systems (Minneapolis, MN). NP-40, phenylmethylsulfonylfluoride, protease inhibitor cocktail, Na_3VO_4, and actin antibody were purchased from Sigma (St. Louis, MO). Antibodies against phospho-eIF4E (Ser-209), eIF4E, phospho-MEK1/2 (Ser-217/221), MEK1/2, phospho-Mnk-1 (Thr-197/202), phospho-S6 kinase (Thr-389), phospho-c-Raf (Ser-259), phospho-Erk1/2 (Thr-202/Tyr-204), Erk1/2, phospho-Akt (Ser-473), S6 kinase, 4EBP-1, and Akt antibodies were obtained from Cell Signaling (Boston, MA). Raf-1, Mnk-1, p85, and Myc antibodies were purchased from Santa Cruz Biotechnology (Delaware, CA). HA antibody was obtained from Covance (Princeton, NJ). Mitogen-activated and extracellular signal-regulated kinase kinase (MEK) inhibitor U0126 was purchased from Promega (Madison, WI). PI 3 kinase inhibitor Ly294002 and mTORC1 inhibitor rapamycin were obtained from Calbiochem (San Diego, CA). Fugene HD transfection reagent was purchased from Roche Molecular Biology (Indianapolis, IN). ^{35}S-methionine was purchased from PerkinElmer (Boston, MA). Adenovirus vector expressing a deletion mutant of the p85 regulatory subunit of PI 3 kinase, which acts as dominant negative enzyme (Ad DN PI 3 K), and adenovirus vector expressing dominant negative Akt (Ad DN Akt) were described previously (Das et al., 2008a, b). Plasmid vectors expressing shRNAs against two independent regions of raptor mRNA and scrambled RNA were described previously (Das et al., 2012). Constitutively active (CA) MEK expression vector was purchased from Stratagene (Santa Clara, CA). Dominant negative Mnk-1 (DN Mnk-1) plasmid was a gift from Dr. J. Cooper, Fred Hutchinson Cancer Research Center, Seattle, WA. Expression vectors containing HA-tagged eIF4E S209A and untagged eIF4E S209D were kindly provided by Dr. H.-G. Wendel, Memorial Sloan-Kettering Cancer Center, NY.

Cell culture and adenovirus infection

Rat and human glomerular mesangial cells were grown in DMEM with 17% and 10% fetal bovine serum, respectively (Das et al., 2008a, b). The cells were infected in serum free medium with respective adenovirus vectors essentially as described previously (Das et al., 2008a, b). Ad GFP, which expresses green fluorescence protein, was used as control for adenovirus vector infection.

Immunoblotting and immunoprecipitation

At the end of incubation, the cells were harvested and lysed in RIPA buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 0.1% protease inhibitor cocktail, and 1% NP-40) at 4°C for 30 min. The cell extracts were centrifuged at 10,000*g* for half an hour at 4°C. The supernatant was used to determine protein concentration using BioRad reagent. Equal amounts of proteins were immunoprecipitated with indicated antibody. The immunebeads or the cell lysates were separated by SDS–polyacrylamide gel electrophoresis followed by transfer to the PVDF membrane. Proteins present in the membrane were immunoblotted with indicated antibodies as described previously (Das et al., 2008a, b).

Transfection

Mesangial cells were seeded at 80% confluency. The following day the cell monolayer was transfected with indicated plasmid expression vector or empty vector using Fugene HD transfection reagent as described previously (Das et al., 2008a, b). The cells were serum-starved for 48 h. 2 ng/ml TGFβ was added for indicated times.

Protein synthesis and hypertrophy

The cells were incubated with ^{35}S-methionine and protein synthesis was determined essentially as described previously (Mahimainathan et al., 2006; Das et al., 2008a). To determine hypertrophy, mesangial cells were trypsinized after incubation, counted using a hemocytometer, then lysed and total protein content determined. Hypertrophy was measured as a ratio of total protein to cell number (Das et al., 2008a).

Statistics

The significance of the data was determined by ANOVA followed by Student–Newman–Keuls analysis as described previously. The means ± SE of indicated measurements are shown. A *P*-value of <0.05 was considered significant.

Results

TGFβ-stimulated Erk1/2 regulates eIF4E phosphorylation and hypertrophy of mesangial cells

We previously showed that TGFβ increases protein synthesis leading to mesangial cell hypertrophy (Das et al., 2008a). Because phosphorylation of eIF4E is required for initiation of cap-dependent mRNA translation (Sonenberg and Hinnebusch, 2007), we tested the effect of TGFβ on eIF4E phosphorylation. TGFβ increased phosphorylation of eIF4E at Ser-209 in a time-dependent manner (Fig. 1A). Mitogen-activated protein kinases including Erk1/2 regulate eIF4E phosphorylation (Morley and McKendrick, 1997). The time course of eIF4E phosphorylation in response to TGFβ correlated with Erk1/2 phosphorylation (Fig. 1A). Because activation of Erk1/2 depends upon its upstream kinase MEK, we tested activation of this kinase using a phospho-specific (Ser-217/221) antibody. TGFβ increased phosphorylation of MEK in a time-dependent manner, which coincided with Erk1/2 activation (Fig. 1B). Moreover, inhibition of MEK with U0126 blocked TGFβ-induced phosphorylation of eIF4E concomitant with attenuation of Erk1/2 phosphorylation (Fig. 1C). Expression of dominant negative Erk2 also prevented phosphorylation of eIF4E (Supplementary Fig. S1).

Next, we tested the involvement of MEK/Erk1/2 in TGFβ-induced protein synthesis. Inhibition of MEK by U0126 decreased TGFβ-induced protein synthesis (Fig. 1D). Since protein synthesis is necessary for hypertrophy of cells, we tested the requirement of MEK in mesangial cell hypertrophy. U0126 significantly inhibited TGFβ-stimulated hypertrophy (Fig. 1E). CA MEK confirmed this observation, where expression of CA MEK increased protein synthesis and hypertrophy of mesangial cells similar to treatment with TGFβ (Supplementary Fig. S2A, B). Because MEK activates Erk1/2, we investigated the role of Erk1/2 in the regulation of mesangial cell hypertrophy. Expression of dominant negative Erk2 suppressed protein synthesis and hypertrophy of mesangial cells in response to TGFβ (Fig. 1F, G, and Supplementary Fig. S3A and B). Collectively the above results suggest that Erk1/2 MAPK regulates phosphorylation of eIF4E and TGFβ-induced mesangial cell hypertrophy.

TGFβ-stimulated PI 3 kinase regulates MEK/Erk-mediated phosphorylation of eIF4E independent of Akt

It has been established that PI 3 kinase controls mesangial cell hypertrophy in response to TGFβ (Mahimainathan et al., 2006). Therefore, we tested the role of PI 3 kinase in MEK/Erk signal transduction. Our results showed that inhibition of Pi 3 kinase by Ly294002 abrogated TGFβ-stimulated phosphorylation of MEK (Fig. 2A). These observation was confirmed by dominant negative PI 3 kinase (Supplementary Fig. S4A). Similarly, both

Ly294002 and dominant negative PI 3 kinase suppressed phosphorylation of Erk1/2 and eIF4E (Fig. 2B, C and Supplementary Fig. S4B, C). These results demonstrate that PI 3 kinase-dependent MEK/Erk1/2 circuit regulates TGFβ-induced phosphorylation of eIF4E in mesangial cells.

MEK/Erk activation is mediated by the upstream kinase Raf-1. Phosphorylation of Raf-1 on Ser-259 by PI 3 kinase target Akt kinase inhibits MEK/Erk pathway (Zimmermann and Moelling, 1999). Since TGFβ activates Akt (Das et al., 2008b), we tested phosphorylation of Raf-1. TGFβ increased phosphorylation of Raf-1 at Ser-259, which was inhibited by Ly294002 and dominant negative PI 3 kinase expression (Fig. 3A and Supplementary Fig. S5). Note that increased Akt-mediated phosphorylation of Raf-1 at Ser-259 by TGFβ did not have any inhibitory effect on MEK/Erk phosphorylation (Figs. 2A, B and 3A). To directly examine the involvement of Akt, we used dominant negative Akt kinase. Although expression of dominant negative Akt inhibited TGFβ-induced Raf-1 phosphorylation (Fig. 3B), it had no effect on TGFβ-stimulated MEK and Erk1/2 phosphorylation (Fig. 3C, D). Furthermore, dominant negative Akt did not affect TGFβ-stimulated phosphorylation of eIF4E (Fig. 3E). These results suggest that in TGFβ-incubated mesangial cells PI 3 kinase-dependent Akt/Raf-1 does not contribute to MEK/Erk1/2 activation and eIF4E phosphorylation.

TGFβ increases phosphorylation of Mnk-1 in PI 3 kinase-dependent manner: Mnk-1 has been shown to be the only kinase that inducibly phosphorylates eIF4E at Ser-209 (Fukunaga and Hunter, 1997; Waskiewicz et al., 1999). We examined the phosphorylation of Mnk-1 in response to TGFβ. TGFβ increased phosphorylation of Mnk-1 in a time-dependent manner (Fig. 4A). MEK inhibitor U0126 blocked TGFβ-stimulated Mnk-1 phosphorylation (Fig. 4B). Also, expression of dominant negative Erk2 significantly suppressed phosphorylation of Mnk-1 in response to TGFβ (Fig. 4C), indicating that Erk 1/2 regulates Mnk-1. As we have shown above that PI 3 kinase regulates MEK/Erk1/2 signal transduction, we tested the sequential role of this lipid kinase in TGFβ signaling. The results showed that inhibition of PI 3 kinase by Ly294002 blocked TGFβ-induced phosphorylation of Mnk-1 (Fig. 4D); similar results were obtained with dominant negative PI 3 kinase (Supplementary Fig. S6).

Mnk-1 regulates TGFβ-induced mesangial cell hypertrophy: Because PI 3 kinase/MEK/Erk1/2 signal transduction regulates mesangial cell hypertrophy and our results above show that this same kinase cascade controls Mnk-1 phosphorylation at Thr-197 and Thr-202 in response to TGFβ, we examined the role of Mnk-1 in hypertrophy of mesangial cells. Expression of dominant negative mutant of Mnk-1 in which Thr-197 and Thr-202 are mutated to alanine (Mnk-1-T2A2) significantly inhibited TGFβ-induced protein synthesis and hypertrophy (Fig. 5A, B). Together our results demonstrate a requirement of Mnk-1 in promoting TGFβ-induced mesangial cell hypertrophy.

Dissociation of eIF4E-4EBP-1 complex is necessary for eIF4E phosphorylation: Phosphorylation of 4EBP-1 by mTORC1 is necessary for its dissociation from eIF4E (Sonenberg and Hinnebusch, 2007). Because TGFβ increases phosphorylation of 4EBP-1 in an mTORC1-dependent manner (Das et al., 2008a), we tested the role of 4EBP-1 dissociation on phosphorylation of eIF4E. Rapamycin suppressed phosphorylation of eIF4E at Ser-209 and inhibited dissociation of eIF4E from 4EBP-1 in TGFβ-treated cells (Fig. 6A, B). Use of two independent shRNAs to downregulate raptor, which is an exclusive component of mTORC1, also suppressed TGFβ-stimulated phosphorylation of eIF4E (Fig. 6C, D). Decreased raptor levels inhibited phosphorylation of S6 kinase at Thr-389, suggesting inhibition of mTORC1 activity (Fig. 6C, D). However, neither rapamycin nor downregulation of raptor inhibited TGFβ-stimulated Mnk-1 phosphorylation (Fig. 6E–G). These data indicate that dissociation of 4EBP-1 is required for Mnk1-mediated

phosphorylation of eIF4E because inhibition of mTORC1 prevented this phosphorylation in the presence of Mnk-1 activation. These data also show that mTORC1 does not affect TGFβ-induced phosphorylation of Mnk-1.

Ser-209 phosphorylation of eIF4E is required for mesangial cell hypertrophy: Dissociation of eIF4E from 4EBP-1 and its phosphorylation may represent a concomitant event in mTORC1 signal transduction for cap-dependent mRNA translation (Sonenberg and Hinnebusch, 2007). However, the requirement of this eIF4E phosphorylation has not been tested in protein synthesis. Therefore, we examined the effect of an eIF4E CA mutant in which the Ser-209 is changed to phospho-mimetic aspartic acid (eIF4E S209D) (Wendel et al., 2007). Mesangial cells transfected with this mutant were treated with TGFβ. Expression of eIF4E S209D by itself significantly increased protein synthesis in a pattern similar to TGFβ (Fig. 7A); however, the extent of increase was greater upon adding TGFβ. Similarly, eIF4E S209D promoted hypertrophy of mesangial cells analogous to that seen with TGFβ (Fig. 7B). To confirm this observation, we used a dominant negative mutant of eIF4E in which the Ser-209 is changed to alanine. Expression of this mutant markedly inhibited TGFβ-stimulated protein synthesis and hypertrophy of mesangial cells (Fig. 7C, D). These results conclusively demonstrate a significant role of eIF4E phosphorylation in TGFβ induction of mesangial cell hypertrophy.

Discussion

In the present study we show that TGFβ increases phosphorylation of eIF4E using a signaling cascade involving PI 3 kinase, MEK and Erk1/2. We demonstrate that this kinase circuit regulates phosphorylation/activation of Mnk-1, which in turn phosphorylates eIF4E at Ser-209, in response to TGFβ. Moreover, we highlight that dissociation of 4EBP-1-eIF4E complex is crucial for Ser-209 phosphorylation of eIF4E. Finally, we demonstrate a requirement of TGFβ-stimulated phosphorylation at Ser-209 residue of eIF4E for mesangial cell protein synthesis and hypertrophy (Fig. 8).

TGFβ enhances protein synthesis by controlling the translational machinery including the rate-limiting initiation step to induce mesangial cell hypertrophy (Das et al., 2008a). eIF4G and 4EBP-1 compete with each other to bind to the same site present in the concave dorsal site of eIF4E (Marcotrigiano et al., 1997). Interestingly, binding of eIF4G with eIF4E increases its capacity to associate with 5′cap structure of the mRNA to initiate translation. TGFβ increases phosphorylation of 4EBP-1 by mTORC1 to release eIF4E to bind eIF4G and form eIF4F complex (Sonenberg and Hinnebusch, 2007; Das et al., 2008a). The Ser-209 phosphorylation site of eIF4E is located in the region of cap-interaction (Marcotrigiano et al., 1997). The requirement of Ser-209 phosphorylation for eIF4E binding to mRNA cap is controversial. For example, eIF4E mutant with acidic residues at Ser-209 showed increased affinity for cap binding (Shibata et al., 1998). In contrast, Scheper et al. found a decrease in binding of Ser-209 phosphorylated eIF4E to the cap analog. Similarly, an acidic mutation of this site showed reduced interaction (Scheper et al., 2002). However, we demonstrate that TGFβ rapidly increased phosphorylation of eIF4E at Ser-209 (Fig. 1A). This phosphorylation is associated with TGFβ-induced dissociation of eIF4E from 4EBP-1 and increase in protein synthesis (Figs. 1D and 6B) (Das et al., 2008a).

Mnk-1 and Mnk-2, which are under the control of Erk1/2, were identified as the direct kinases for eIF4E Ser-209 phosphorylation (Fukunaga and Hunter, 1997). The basal phosphorylation of eIF4E was abolished in mouse embryonic fibroblasts lacking Mnk-2. Similarly, eIF4E phosphorylation was attenuated in various organs in Mnk-2 null mouse (Ueda et al., 2004). In contrast, studies with Mnk-1 null mouse fibroblasts and different tissues from Mnk-1−/− mouse showed that this kinase was required for inducible

phosphorylation of eIF4E (Ueda et al., 2004). In conjunction with these results we demonstrate that TGFβ increased eIF4E Ser-209 phosphorylation by Mnk-1 in an Erk1/2-sensitive manner (Figs. 1 and 4A–C).

Growth factor receptors and cytokines activate two signal transduction pathways: PI 3 kinase/Akt and Ras/MAPK. We and others have shown previously that TGFβ activates both these kinase cascades (Bakin et al., 2000; Ghosh Choudhury and Abboud, 2004). Although independent mechanisms exist for activation of these kinase pathways, a cross-talk between Ras and PI 3 kinase has been previously reported (Rodriguez-Viciana et al., 1996). In mesangial cells, we found that PI 3 kinase regulated MEK/Erk activation in response to TGFβ (Fig. 2). MEK/Erk pathway is regulated by its upstream kinase Raf-1. But PI 3 kinase/Akt-mediated phosphorylation of Raf-1 at Ser-259 negatively regulates MEK/Erk (Zimmermann and Moelling, 1999). Accordingly, we show increased phosphorylation of Raf-1 by TGFβ. This may be due to activation of Akt via PI 3 kinase, which we reported previously (Ghosh Choudhury and Abboud, 2004). In fact we show attenuation of Raf-1 phosphorylation by PI 3 kinase inhibition (Fig. 3A). Interestingly, inhibitory phosphorylation of Raf-1 did not affect activation of MEK and Erk1/2 in the presence of TGFβ (Figs. 2 and 3C, D). Furthermore, our results demonstrate that downstream of PI 3 kinase, Akt does not contribute to activation of MEK/Erk, which regulates phosphorylation of eIF4E (Fig. 3C–E).

Mnk-1-mediated eIF4E phosphorylation is concomitant with mTORC1-phosphorylated 4EBP-1 and its dissociation from 4EBP-1-eIF4E complex (Sonenberg and Hinnebusch, 2007). Mnk has high affinity for C-terminal region of eIF4G. Therefore, it was postulated that phosphorylation of eIF4E may occur after eIF4E-eIF4G binding (Hay, 2010). In fact, we show that rapamycin, which inhibits TGFβ-induced dissociation of eIF4E from the 4EBP-1-eIF4E complex, blocked phosphorylation of eIF4E at Ser-209 (Fig. 6A, B). Since Mnk-1 phosphorylates eIF4E at this site, one possibility is that rapamycin may block phosphorylation/activation of Mnk-1. Our results with rapamycin and sh-raptor do not support this notion as indicated by the failure of inhibition of mTORC1 to block TGFβ-stimulated Mnk-1 phosphorylation (Fig. 6E–G). These data conclusively demonstrate that in the context of TGFβ-induced protein synthesis in mesangial cells, dissociation of eIF4E is necessary for TGFβ-stimulated increase in phosphorylation of eIF4E.

The functional consequences of Mnk-mediated phosphorylation of eIF4E have been debated, as Mnk-1/2 double knock out mouse is viable with normal cap-dependent protein synthesis (Ueda et al., 2004). It is possible that function of Mnk may be necessary under conditions of cellular insult. In support of this notion, our data demonstrate that expression of dominant negative Mnk-1 significantly inhibited protein synthesis and cellular hypertrophy in response to TGFβ (Fig. 5).

The biological significance of phosphorylation of eIF4E is debated as unphosphorylated eIF4E could increase mRNA translation in vitro and could form eIF4F complex in insulin-stimulated cells (Scheper and Proud, 2002). However, when the mammalian equivalent of eIF4E serine phosphorylation site in *Drosophila* eIF4E (Ser-251) was mutated to alanine, the transgenic fly showed reduced viability and 35% lethality (Lachance et al., 2002). Although increased phosphorylation of eIF4E at Ser-209 is linked to development of cancer, its requirement was studied indirectly using lethally irradiated mouse model with hematopoietic stem cell-transduced mutant eIF4E Ser-209A, which showed reduced tumorigenicity in these animals (Wendel et al., 2007). More recently, using a specific knock-in mouse model of nonphosphorylatable eIF4E S209A mutant showed requirement of eIF4E phosphorylation for prostate cancer development, which required upregulation of mRNA translation (Furic et al., 2010). In the present study, using the same mutant, we demonstrate

a requirement of eIF4E Ser-209 phosphorylation for TGFβ-stimulated protein synthesis and hypertrophy (Fig. 7C, D). We further validate this observation using phospho-mimetic eIF4E S209D mutant (Fig. 7A, B). To our knowledge, these results represent the first demonstration of Mnk-1-mediated phosphorylation of eIF4E in mediation of cellular hypertrophy. Recent data demonstrated beneficial effects of rapamycin in models of renal disease such as diabetes, where TGFβ significantly contributes to kidney hypertrophy. Targeting Mnk-1 and/or eIF4E may produce major impact on progression of the disease process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Jeff Barnes for critically reading the manuscript. This work was supported by the NIH RO1 DK50190 and VA Merit Review grants to G.G.C. G.G.C. is a recipient of VA Senior Research Career Scientist Award. N.G.C. is supported by NIH RO1 AR52425 and VA Merit Review grants. B.S.K. is supported by grants from NIH (RO1 DK077295 and RC2A 036613) and VA Research Service.

Literature Cited

Abboud HE. Mesangial cell biology. Exp Cell Res. 2012; 318:979–985. [PubMed: 22414873]

Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL. Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. J Biol Chem. 2000; 275:36803–36810. [PubMed: 10969078]

Bottinger EP. TGF-beta in renal injury and disease. Semin Nephrol. 2007; 27:309–320. [PubMed: 17533008]

Das F, Ghosh-Choudhury N, Mahimainathan L, Venkatesan B, Feliers D, Riley DJ, Kasinath BS, Choudhury GG. Raptor-rictor axis in TGFbeta-induced protein synthesis. Cell Signal. 2008a; 20:409–423. [PubMed: 18068336]

Das F, Ghosh-Choudhury N, Venkatesan B, Li X, Mahimainathan L, Choudhury GG. Akt kinase targets association of CBP with SMAD 3 to regulate TGFbeta-induced expression of plasminogen activator inhibitor-1. J Cell Physiol. 2008b; 214:513–527. [PubMed: 17671970]

Das F, Ghosh-Choudhury N, Dey N, Mandal CC, Mahimainathan L, Kasinath BS, Abboud HE, Choudhury GG. Unrestrained mammalian target of rapamycin complexes 1 and 2 increase expression of phosphatase and tensin homolog deleted on chromosome 10 to regulate phosphorylation of akt kinase. J Biol Chem. 2012; 287:3808–3822. [PubMed: 22184110]

Eddy AA, Neilson EG. Chronic kidney disease progression. J Am Soc Nephrol. 2006; 17:2964–2966. [PubMed: 17035605]

Fukunaga R, Hunter T. MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. EMBO J. 1997; 16:1921–1933. [PubMed: 9155018]

Furic L, Rong L, Larsson O, Koumakpayi IH, Yoshida K, Brueschke A, Petroulakis E, Robichaud N, Pollak M, Gaboury LA, Pandolfi PP, Saad F, Sonenberg N. eIF4E phosphorylation promotes tumorigenesis and is associated with prostate cancer progression. Proc Natl Acad Sci USA. 2010; 107:14134–14139. [PubMed: 20679199]

Ghosh Choudhury G, Abboud HE. Tyrosine phosphorylation-dependent PI 3 kinase/Akt signal transduction regulates TGFbeta-induced fibronectin expression in mesangial cells. Cell Signal. 2004; 16:31–41. [PubMed: 14607273]

Hay N. Mnk earmarks eIF4E for cancer therapy. Proc Natl Acad Sci USA. 2010; 107:13975–13976. [PubMed: 20679238]

Iwano M, Kubo A, Nishino T, Sato H, Nishioka H, Akai Y, Kurioka H, Fujii Y, Kanauchi M, Shiiki H, Dohi K. Quantification of glomerular TGF-beta 1 mRNA in patients with diabetes mellitus. Kidney Int. 1996; 49:1120–1126. [PubMed: 8691733]

Kopp JB, Factor VM, Mozes M, Nagy P, Sanderson N, Bottinger EP, Klotman PE, Thorgeirsson SS. Transgenic mice with increased plasma levels of TGF-beta 1 develop progressive renal disease. Lab Invest. 1996; 74:991–1003. [PubMed: 8667617]

Lachance PE, Miron M, Raught B, Sonenberg N, Lasko P. Phosphorylation of eukaryotic translation initiation factor 4E is critical for growth. Mol Cell Biol. 2002; 22:1656–1663. [PubMed: 11865045]

Mahimainathan L, Das F, Venkatesan B, Choudhury GG. Mesangial cell hypertrophy by high glucose is mediated by downregulation of the tumor suppressor PTEN. Diabetes. 2006; 55:2115–2125. [PubMed: 16804083]

Marcotrigiano J, Gingras AC, Sonenberg N, Burley SK. Cocrystal structure of the messenger RNA 5′ cap-binding protein (eIF4E) bound to 7-methyl-GDP. Cell. 1997; 89:951–961. [PubMed: 9200613]

Morley SJ, McKendrick L. Involvement of stress-activated protein kinase and p38/RK mitogen-activated protein kinase signaling pathways in the enhanced phosphorylation of initiation factor 4E in NIH 3T3 cells. J Biol Chem. 1997; 272:17887–17893. [PubMed: 9211946]

Rodriguez-Viciana P, Warne PH, Vanhaesebroeck B, Waterfield MD, Downward J. Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation. EMBO J. 1996; 15:2442–2451. [PubMed: 8665852]

Scheper GC, Proud CG. Does phosphorylation of the cap-binding protein eIF4E play a role in translation initiation? Eur J Biochem. 2002; 269:5350–5359. [PubMed: 12423333]

Scheper GC, van Kollenburg B, Hu J, Luo Y, Goss DJ, Proud CG. Phosphorylation of eukaryotic initiation factor 4E markedly reduces its affinity for capped mRNA. J Biol Chem. 2002; 277:3303–3309. [PubMed: 11723111]

Shi Y, Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell. 2003; 113:685–700. [PubMed: 12809600]

Shibata S, Morino S, Tomoo K, In Y, Ishida T. Effect of mRNA cap structure on eIF-4E phosphorylation and cap binding analyses using Ser209-mutated eIF-4Es. Biochem Biophys Res Commun. 1998; 247:213–216. [PubMed: 9642105]

Sonenberg N, Hinnebusch AG. New modes of translational control in development, behavior, and disease. Mol Cell. 2007; 28:721–729. [PubMed: 18082597]

Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL. SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. Cell. 1998; 95:779–791. [PubMed: 9865696]

Ueda T, Watanabe-Fukunaga R, Fukuyama H, Nagata S, Fukunaga R. Mnk2 and Mnk1 are essential for constitutive and inducible phosphorylation of eukaryotic initiation factor 4E but not for cell growth or development. Mol Cell Biol. 2004; 24:6539–6549. [PubMed: 15254222]

Waskiewicz AJ, Johnson JC, Penn B, Mahalingam M, Kimball SR, Cooper JA. Phosphorylation of the cap-binding protein eukaryotic translation initiation factor 4E by protein kinase Mnk1 in vivo. Mol Cell Biol. 1999; 19:1871–1880. [PubMed: 10022874]

Wendel HG, Silva RL, Malina A, Mills JR, Zhu H, Ueda T, Watanabe-Fukunaga R, Fukunaga R, Teruya-Feldstein J, Pelletier J, Lowe SW. Dissecting eIF4E action in tumorigenesis. Genes Dev. 2007; 21:3232–3237. [PubMed: 18055695]

Zimmermann S, Moelling K. Phosphorylation and regulation of Raf by Akt (protein kinase B). Science. 1999; 286:1741–1744. [PubMed: 10576742]

Ziyadeh FN, Hoffman BB, Han DC, Iglesias-De La Cruz MC, Hong SW, Isono M, Chen S, McGowan TA, Sharma K. Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal anti transforming growth factor-beta antibody in db/db diabetic mice. Proc Natl Acad Sci USA. 2000; 97:8015–8020. [PubMed: 10859350]

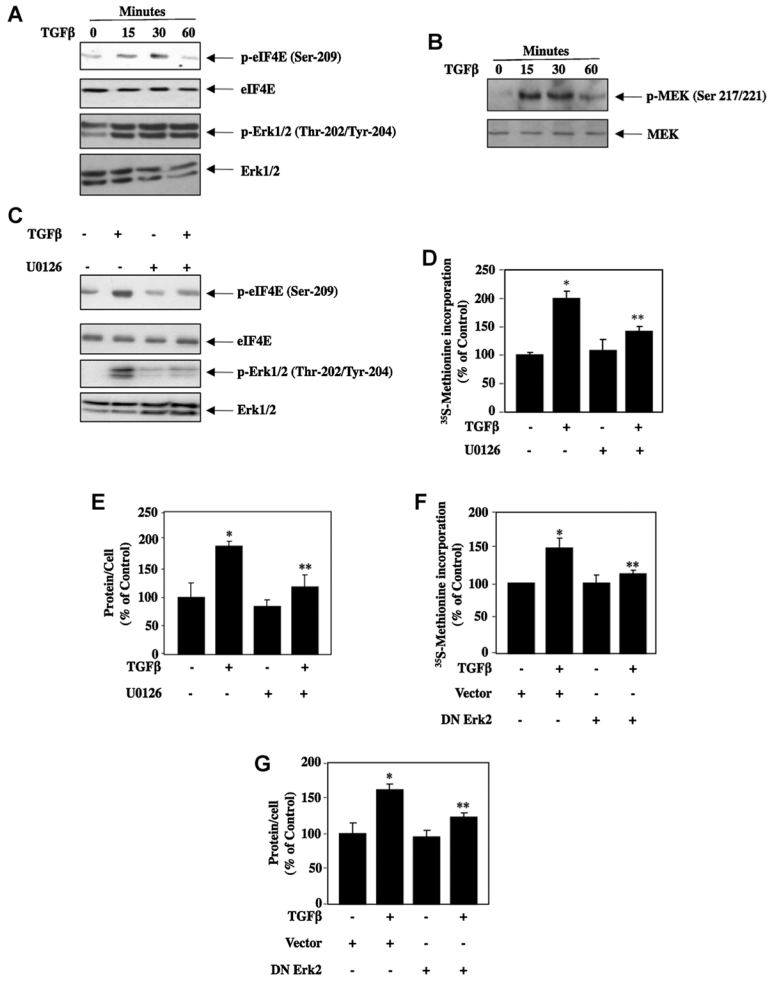

Fig. 1.

TGFβ increases phosphorylation of eIF4E and mesangial cell hypertrophy in Erk1/2-dependent manner. A, B: Serum-starved (48 h) mesangial cells were incubated with 2 ng/ml TGFβ for the indicated times. The cell lysates were immunoblotted with phospho-eIF4E, eIF4E, phospho-Erk1/2, Erk1/2 (part A), and phospho-MEK and MEK (part B) antibodies. C: Mesangial cells were treated with 5 μM U0126 for 1 h prior to incubation with 2 ng/ml TGFβ for 15 min. The cell lysates were immunoblotted with the indicated antibodies. D, E: Serum-starved (24 h) mesangial cells were incubated with 5 μM of U0126 for last 1 h. TGFβ was added for 24 h. Protein synthesis (part D) and hypertrophy (part E) were determined as described (Das et al., 2008a). In part E, cell hypertrophy was determined as increase in the ratio of total protein to cell number as described (Das et al., 2008a). In part D, $^{*}P < 0.01$ versus control; $^{**}P < 0.05$ versus TGFβ-stimulated. In part E, $^{*}P < 0.05$ versus control; $^{**}P < 0.05$ versus TGFβ-stimulated. F, G: Mesangial cells were transfected with dominant negative Myc Erk2. Transfected cells were serum starved for 24 h and then incubated with 2 ng/ml TGFβ for 24 h. Protein synthesis (part F) and hypertrophy (part G) were determined as described above. Mean ±SE of triplicate measurements is shown. For part F, $^{*}P < 0.05$ versus control; $^{**}P < 0.05$ versus TGFβ-stimulated. For part G, $^{*}P < 0.01$ versus control; $^{**}P < 0.05$ versus TGFβ-stimulated. Bottom parts show expression of Myc Erk2 and actin in one representative samples.

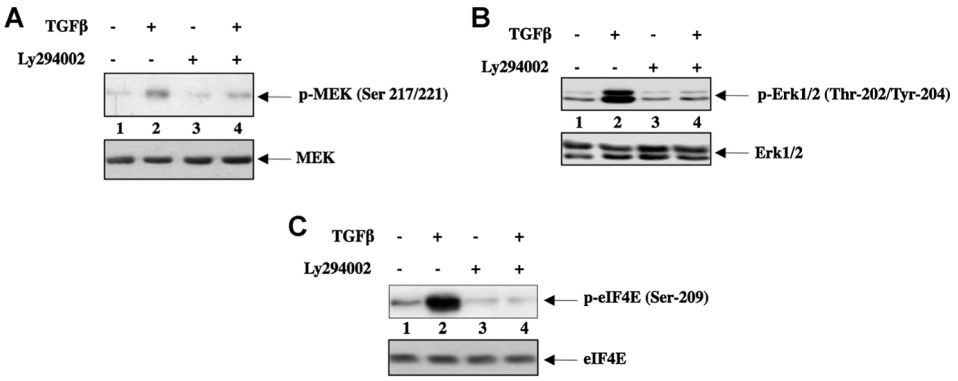

Fig. 2.

PI 3 kinase regulates Erk1/2 dependent phosphorylation of eIF4E. Forty hours serum-starved mesangial cells were treated with 25 μM Ly294002 prior to incubation with 2 ng/ml TGFβ for 15 min. The cell lysates were immunoblotted with antibodies against phospho-MEK, MEK (part A), phospho-Erk1/2, Erk1/2 (part B), and phospho-eIF4E, eIF4E (part C).

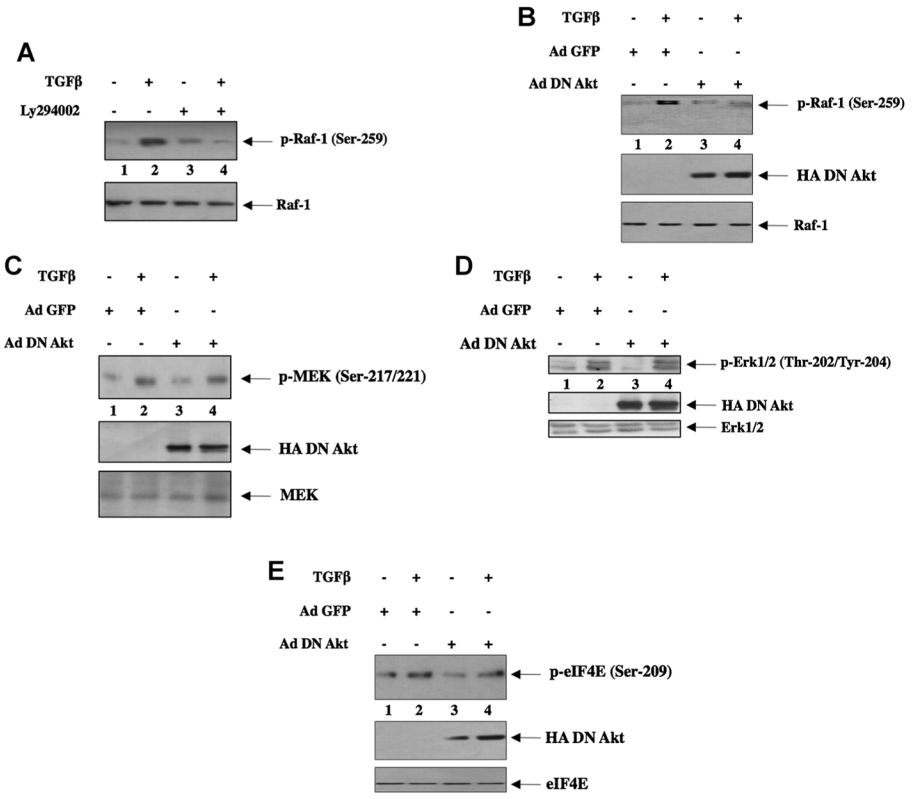

Fig. 3.

PI 3 kinase regulates Raf-1 phosphorylation in the absence of Akt-mediated Erk1/2 and eIF4E phosphorylation. A: Mesangial cells were serum-starved for 48 h and treated with 25 μM Ly294002 for 1 h followed by incubation with 2 ng/ml TGFβ for 15 min. The cell lysates were immunoblotted with phospho-Raf-1 (Ser-259) and Raf-1 antibodies. B–E: Mesangial cells were infected with adenovirus vector expressing dominant negative Akt (Ad DN Akt) for 24 h in serum-free medium. The infected cells were further serum-starved for 24 h and incubated with 2 ng/ml TGFβ for 15 min. The cell lysates were immunoblotted with indicated antibodies.

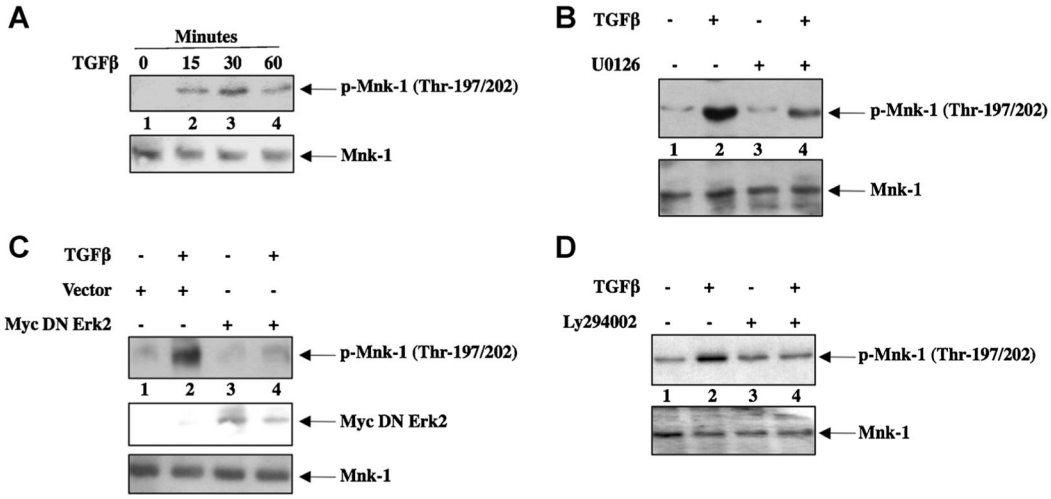

Fig. 4.

Erk1/2 and PI 3 kinase regulate TGFβ-stimulated phosphorylation of Mnk-1. A: Forty-eight hours serum-starved mesangial cells were incubated with 2 ng/ml TGFβ for the indicated times. The cell lysates were immunoblotted with phospho-Mnk-1 and Mnk-1 antibodies. B,D: Mesangial cells were incubated with 5 μMU0126 (part B) and 25 μMLy294002 (part D) for 1 h. The cells were then incubated with 2 ng/ml TGFβ for 15 min. The cell lysates were immunoblotted with phospho-Mnk-1 and Mnk-1 antibodies. Part C: Mesangial cells were transfected with empty vector or dominant negative Myc-tagged Erk2 (DN Myc Erk2). The serum-starved cells were then incubated with 2 ng/ml TGFβ for 15 min. The cell lysates were immunoblotted with phospho-Mnk-1, Myc, Mnk-1 antibodies as indicated.

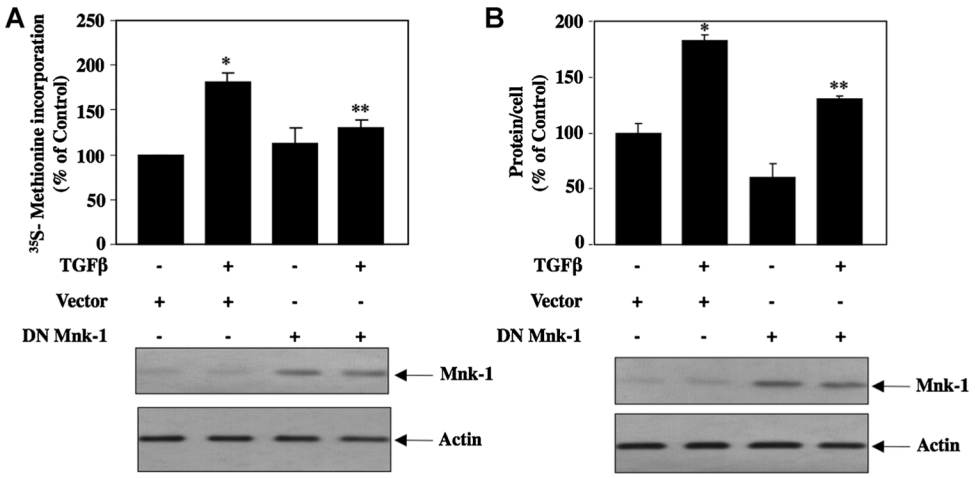

Fig. 5.

Requirement of Mnk-1 for TGFβ-induced protein synthesis and hypertrophy. Mesangial cells were transfected with dominant negative Mnk-1 plasmid or empty vector (control) and serum-starved for 24 h. To these cells2 ng/ml TGFβ was added for 24 h. Protein synthesis (part A) and hypertrophy (part B) were measured as described (Das et al., 2008a). The bottom parts show expression of Mnk-1 and actin in representative samples.

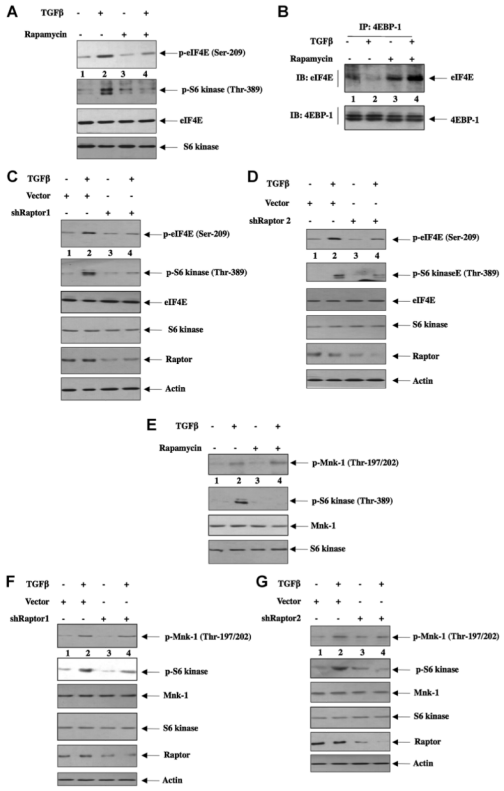

Fig. 6.

Dissociation of eIF4E-4EBP-1 complex is necessary for eIF4E phosphorylation. A, E: Serum-starved mesangial cells were treated with 25 nM rapamycin prior to incubation with 2 ng/ml TGFβ for 15 min. The cell lysates were immunoblotted with phospho-eIF4E, phospho-S6 kinase, eIF4E, S6 kinase (part A), phospho-Mnk-1, and Mnk-1 (part E) antibodies as indicated. B: Lysates of mesangial cells treated with rapamycin and TGFβ similar to as described in parts A and E were immunoprecipitated with 4EBP-1 antibody followed by immunoblotting with eIF4E antibody. C, D, F, G: Mesangial cells were transfected with vector expressing scrambled RNA or shRNA against raptor (shRaptor 1, parts C and F) (shRaptor 2, parts D and G). The transfected cells were serum-starved for 48 hand incubated with 2 ng/ml TGFβ for 15 min. The cell lysates were immunoblotted with phospho-eIF4E, phospho-S6 kinase, eIF4E, S6 kinase, phospho-Mnk-1, Mnk-1, raptor, and actin antibodies as indicated.

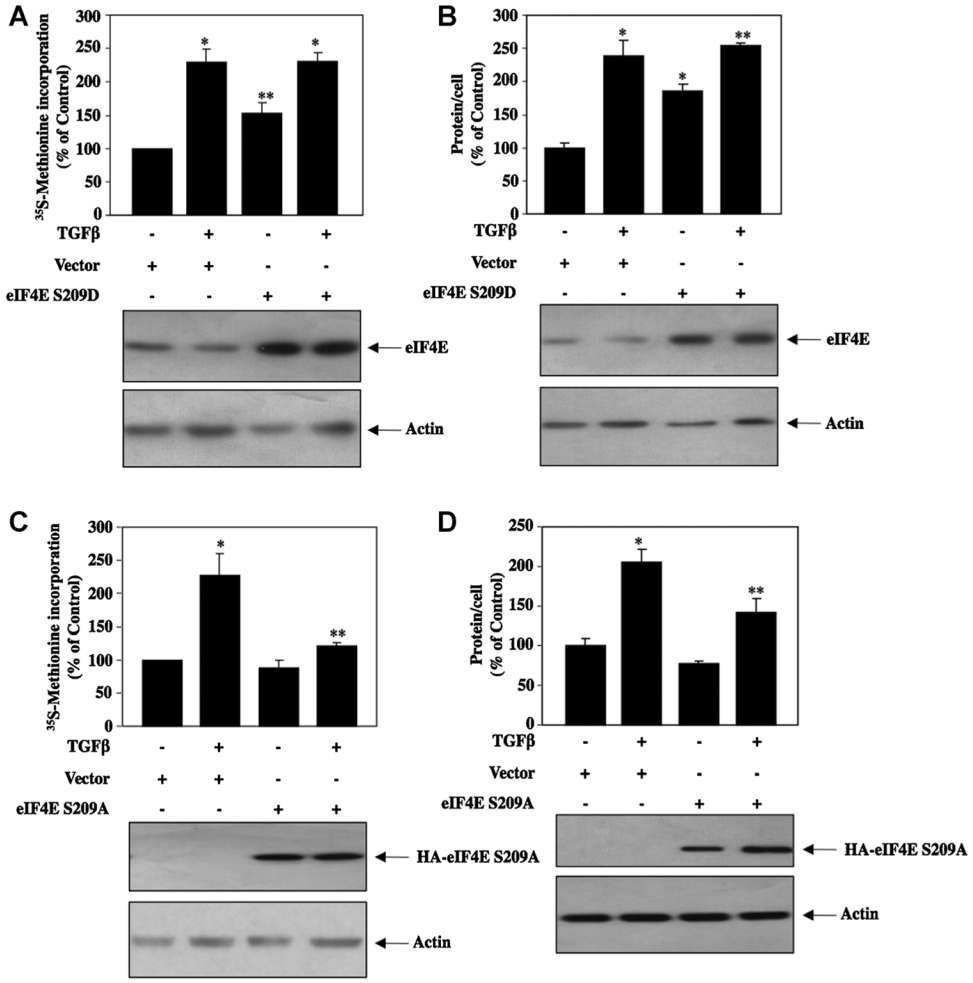

Fig. 7.

Phosphorylation of eIF4E is required for protein synthesis and hypertrophy. A, B: Mesangial cells were transfected with eIF4E S209D phospho-mimetic mutant. The transfected cells serum-starved for 24 h and then incubated with 2 ng/ml TGFβ for 24 h. Protein synthesis (part A) and hypertrophy (part B) were measured as described in Figure 2. Mean ±SE of triplicate measurements is shown. In part A, $^{*}P < 0.001$ versus control; $^{**}P < 0.05$ versus control. In part B, $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus control. C, D: Mesangial cells were transfected with eIF4E S209A phospho-deficient mutant. The transfected cells were starved and incubated with 2 ng/ml TGFβ as described above. Protein synthesis (part C) and hypertrophy (part D) were measured as described in Figure 2. Mean ±SE of triplicate measurements is shown. In part C, $^{*}P < 0.01$ versus control; $^{**}P < 0.01$ versus TGFβ-stimulated. In part D, $^{*}P < 0.001$ versus control; $^{**}P < 0.05$ versus TGFβ-stimulated.

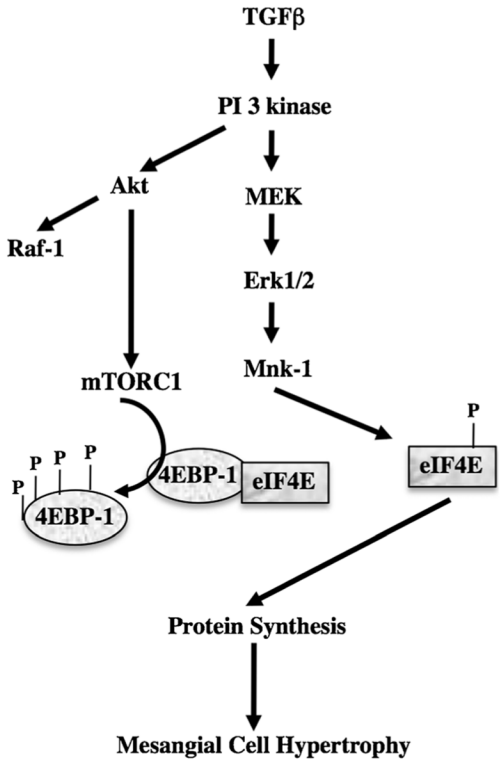

Fig. 8.
Schematic summarizing our results.