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TGF β enforces activation of eukaryotic elongation factor-2 (eEF2) via inactivation of eEF2 kinase by p90 ribosomal S6 kinase (p90Rsk) to induce mesangial cell hypertrophy

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Abstract

eEF2 phosphorylation is under tight control to maintain mRNA translation elongation. We report that TGF β activates eEF2 by decreasing eEF2 phosphorylation and simultaneously increasing eEF2 kinase phosphorylation. Remarkably, inhibition of Erk1/2 blocked the TGF β -induced dephosphorylation and phosphorylation of eEF2 and eEF2 kinase. TGF β increased phosphorylation of p90Rsk in an Erk1/2-dependent manner. Inactive p90Rsk reversed TGF β -inhibited phosphorylation of eEF2 and suppressed eEF2 kinase activity. Finally, inactive p90Rsk significantly attenuated TGF β -induced protein synthesis and hypertrophy of mesangial cells. These results present the first evidence that TGF β utilizes the two layered kinase module Erk/p90Rsk to activate eEF2 for increased protein synthesis during cellular hypertrophy.

Keywords

Diabetic nephropathy; Receptor serine/threonine kinase; mRNA translation

1. Introduction

Transforming growth factor- β (TGF β) is an enforcer of diverse functions including immune regulation, wound healing, cell proliferation and migration, apoptosis and extracellular matrix expansion [1]. The active TGF β dimer elicits these biological activities by binding to type II receptor, which phosphorylates the type I receptor at GS domain, a 30 amino acid segment, located immediately upstream of the kinase domain [1]. Activated type I receptor in the tetrameric complex recruits the TGF β receptor-specific R-Smads2/3 to phosphorylate at the C-terminal serine residues. Phosphorylated Smads dissociate from the cytoplasmic retention protein SARA to expose the nuclear localization signal and translocate to the

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nucleus and associate with common Smad, Smad4 [2]. The heterodimeric Smad complex then associates with other DNA binding transcription factors and transcriptional coactivator or corepressor to induce or repress gene expression [1,2]. However, Smad-independent TGF β signaling also coexists with the canonical Smad pathway. These pathways include Erk1/2, JNK and p38 MAPK [1]. We and others have recently shown that TGF β activates phosphatidylinositol 3 kinase/Akt cascade to increase expression of collagen I and fibronectin, two profibrotic genes [3,4].

TGF β contributes to nephropathy, which consists of whole kidney and glomerular hypertrophy and altered glomerular hemodynamics [5]. Increased glomerular volume correlates with hypertrophy of mesangial cells, which constitute a third of the glomerular cell population that leads to accumulation of matrix proteins and glomerulosclerosis [6]. We have recently reported involvement of TGF β -induced PI 3 kinase/Akt signaling in the development of glomerular, especially mesangial cell hypertrophy, during the progression of diabetic nephropathy [7].

Cellular hypertrophy is defined by the increase in protein synthesis with minimal changes in DNA synthesis [8]. About 95% of mRNAs are translated in a cap-dependent manner, where translation initiation phase acts as the rate-limiting step [9]. We have recently demonstrated a role of TGF β -stimulated TORC1 activity in inactivation of the translation repressor 4EBP-1, which contributes to formation of initiation complex 4F by releasing the eIF4E to induce cap-dependent mRNA translation [9,10]. However, it is during the elongation phase that peptide synthesis occurs in both cap-dependent and -independent translation. The 93 kD eukaryotic elongation factor 2 (eEF2), when bound to GTP in its N-terminal segment, mediates the translocation of aminoacyl tRNA during elongation [11,12]. Phosphorylation of threonine-56 in this domain inhibits its ribosome binding activity thereby inactivating it [11]. eEF2 kinase (eEF2K) that phosphorylates eEF2 at Thr-56 is unusual in that it is not a member of the Ser-Thr-Tyr kinase superfamily [13]. The N-terminus of eEF2K contains a calmodulin binding site and its activity is normally dependent upon Ca²⁺ and calmodulin [11]. Several serine residues including Ser-366 have been identified in the eEF2K, which are phosphorylated by different stimuli that specifically activate kinases such as S6 kinase, Rsk1/2, SAPK4, CDC2, PKA and MK-2 [11,14,15]. Insulin- and IGF-1-mediated activation of receptor tyrosine kinases induces phosphorylation of eEF2K at Ser-366, which impairs its activity towards eEF2, resulting in increased elongation of protein synthesis [11,16]. How eEF2 phosphorylation is regulated by eEF2K in response to serine-threonine kinase receptor activation is not known.

In the present study, we demonstrate that TGF β induces dephosphorylation of eEF2 with concomitant increase in phosphorylation of eEF2K. These phosphorylation events are sensitive to activation of Erk1/2 and its target p90 ribosomal S6 kinase (Rsk). Finally, we demonstrate that TGF β -stimulated p90Rsk contributes to mesangial cell protein synthesis and hypertrophy.

2. Materials and Methods

2.1. Materials

Recombinant TGF β was purchased from R & D Systems. Phospho-eEF2 (Thr-56), eEF2, phospho-eEF2K (Ser-366), eEF2K, phospho-Erk1/2 (Thr-202/Tyr-204), Erk1/2, phospho-p90Rsk (Ser-380) and p90Rsk antibodies were obtained from Cell Signaling Technology. Anti-actin antibody was obtained from Sigma. Anti-HA antibody was purchased from Covance. Dominant negative Erk2 expression vector has been described previously [17]. Expression of dominant negative Erk2 blocks the kinase activity of both Erk1 and Erk2 [18]. Dominant negative p90Rsk1 plasmid (pKH3 Rsk1 K112/464R) was kindly provided by Dr.

John Blenis, Harvard Medical School. Dominant negative Rsk1 may inhibit the kinase activity of other Rsk isoforms. However, Rsk1 is predominantly expressed in kidney [19].

2.2. Cell Culture and transfection

Rat kidney glomerular mesangial cells were grown as described previously [4,7,10]. Mesangial cells were transfected with vector or indicated expression plasmids using Fugene as described [10,20,21]. Using this reagent, we routinely observe 60 – 80% transfection efficiency in mesangial cells [10].

2.3. Immunoblotting

Mesangial cells were lysed in RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na₃VO₄, 1 mM PMSF, 0.1% protease inhibitor cocktail and 1% NP-40) and the cell debris were separated by centrifugation to yield supernatant, which was used to determine protein concentration as described [4,7,10,20]. Equal amounts of proteins were separated by SDS polyacrylamide gel electrophoresis followed by transfer of the separated proteins to PVDF membrane for immunoblotting with indicated antibodies as described [4,7,10,20].

2.4. Protein synthesis assay and measurement of mesangial cell hypertrophy

Protein synthesis was determined using ³⁵S-methionine incorporation as described [7,10,21]. The cellular hypertrophy is defined by increase in protein synthesis without minimal changes in DNA synthesis, i.e., in the absence of cell proliferation. Therefore, hypertrophy of mesangial cells was measured as the ratio of total protein to number of cells as described [7,10,21].

3. Results

3.1. TGF β activates eEF2 in Erk1/2-sensitive manner

The phosphorylation of eEF2 at Thr-56 inactivates its function to induce elongation phase of mRNA translation [11,12]. To study eEF2 phosphorylation in response to TGF β , we immunoblotted lysates of TGF β -incubated mesangial cells with phospho-eEF2 antibody. TGF β decreased eEF2 phosphorylation in a time-dependent manner (Fig. 1A and Supplemental Fig. S1A). The eEF2 phosphorylation at Thr-56 is mediated by the highly specific eEF2K; activity of eEF2K is impaired by phosphorylation at Ser-366 [11]. Incubation of mesangial cells with TGF β significantly increased phosphorylation of eEF2K (Fig. 1B and Supplemental Fig. S1B). Note that, dephosphorylation of eEF2 followed the same kinetics as that of eEF2K phosphorylation and hence inactivation (Figs. 1A and 1B).

TGF β is known to activate the non-Smad signaling, including Erk1/2 [1]. TGF β increased phosphorylation of Erk1/2 in mesangial cells (Supplementary Fig. S2). We examined the involvement of Erk1/2 in eEF2 phosphorylation using its upstream kinase MEK inhibitor U0126. U0126 significantly blocked the inhibition of phosphorylation of eEF2 in response to TGF β (Fig. 1C and Supplemental Fig. S1C). Furthermore, MEK inhibitor prevented TGF β -stimulated phosphorylation of eEF2K (Fig. 1D and Supplemental Fig. S1D). To confirm the role of Erk, we used dominant negative Erk2. Expression of dominant negative Erk2 prevented the inhibition of phosphorylation of eEF2 (Supplemental Fig. S3A). Additionally, kinase dead Erk2 attenuated the phosphorylation of eEF2K (Supplemental Fig. S3B). These results indicate that following TGF β stimulation, Erk signal transduction pathway regulates the eEF2 dephosphorylation/activation via phosphorylation/inactivation of eEF2K.

3.2 TGF β increases phosphorylation of p90Rsk

The Thr-366 of eEF2K represents a consensus phosphorylation site, which is recognized by many basophilic kinases such as p90Rsk [19]. Furthermore, p90Rsk is phosphorylated and activated downstream of Erk1/2 by activated receptor tyrosine kinases [19]. Activated p90Rsk undergoes autophosphorylation at Ser-380 [19]. Therefore, phosphorylation of p90Rsk at Ser-380 serves as surrogate for activation of this enzyme. Activation of serine/threonine kinase receptor by TGF β in mesangial cells increased phosphorylation of p90Rsk at Ser-380 in a time-dependent manner (Fig. 2A and Supplemental Fig. S4A). U0126, which inhibits activation of Erk1/2, significantly attenuated the TGF β -stimulated phosphorylation of p90Rsk (Fig. 2B and Supplemental Fig. S4B). Furthermore, expression of dominant negative Erk2 significantly inhibited p90Rsk phosphorylation (Fig. 2C and Supplemental Fig. S4C). These results demonstrate that TGF β activates p90Rsk in Erk1/2-dependent manner.

3.3. TGF β -stimulated p90Rsk regulates phosphorylation of eEF2K

Involvement of p90Rsk in TGF β -induced phosphorylation of eEF2 was first examined using a dominant negative p90Rsk kinase in mesangial cells (Supplementary Fig. S5). Expression of dominant negative p90Rsk significantly prevented TGF β -induced inhibition of eEF2 phosphorylation (Fig. 3A and Supplemental Fig. S6A). Furthermore, inactive p90Rsk significantly inhibited the phosphorylation of eEF2K in response to TGF β . (Fig. 3B and Supplemental Fig. S6B). These results indicate that inhibition of the TGF β -stimulated inactivating phosphorylation of eEF2K in the presence of dominant negative p90Rsk results in reversal of TGF β -induced reduction of phosphorylation of eEF2.

3.4. p90Rsk regulates TGF β -induced protein synthesis and mesangial cell hypertrophy

On the basis of our results above, we hypothesized that the p90Rsk governs the elongation phase of mRNA translation by inactivation of eEF2K to induce reduced phosphorylation and activation of eEF2, which results in protein synthesis. We examined the role of p90Rsk on TGF β -induced protein synthesis in mesangial cells. TGF β markedly increased protein synthesis (Fig. 4A). Expression of dominant negative p90Rsk significantly inhibited TGF β -induced protein synthesis (Fig. 4A). Increase in protein synthesis by TGF β causes hypertrophy of mesangial cells [7,10]. As expected, TGF β induced significant hypertrophy in mesangial cells (Fig. 4B). Expression of dominant negative p90Rsk significantly inhibited TGF β -stimulated hypertrophy (Fig. 4B). These results indicate a critical role of p90Rsk in TGF β -induced mesangial cell protein synthesis and hypertrophy.

4. Discussion

The present study explored a non-Smad signaling pathway induced by TGF β to elucidate the contribution of the elongation phase of mRNA translation to mesangial cell hypertrophy. We have delineated a molecular pathway by which TGF β -stimulated Erk1/2 reduced the phosphorylation of eEF2 due to inactivating phosphorylation of eEF2K. We identified p90Rsk as the kinase downstream of Erk1/2 that deactivates eEF2K to cause reduced phosphorylation and activation of eEF2 in response to TGF β . Finally, we provide evidence that TGF β -stimulated p90Rsk activity contributes to protein synthesis, resulting in mesangial cell hypertrophy.

In renal cells the profibrotic action of TGF β precedes hypertrophy. For example, in diabetic nephropathy, the matrix amassing effect of hyperglycemia is partly mediated by TGF β . However, glomerular, especially mesangial cell hypertrophy is followed by matrix protein expansion. Renal cells isolated from TGF β null mice displayed reduced hypertrophy in response to high glucose [22]. Moreover, type 1 diabetes induced in TGF β receptor II

heterozygous mice exhibited reduced glomerular, specifically, mesangial cell hypertrophy [23]. We have recently reported a significant role of mTOR, especially of TORC1 in the induction of mesangial cell hypertrophy in response to TGF β [10]. This action of TORC1 was mediated by inactivation of 4EBP-1, which represses the initiation of cap-dependent mRNA translation [9]. However, irrespective of the mode of initiation, elongation phase of mRNA translation significantly contributes to protein synthesis to induce cellular hypertrophy.

Dephosphorylation of eEF2 induces binding to and facilitates translocation of mRNA on the ribosome to cause elongation of mRNA translation [11]. Insulin in Chinese hamster ovary cells and in primary adipocytes and myocytes decreases eEF2 phosphorylation in a rapamycin-sensitive manner, indicating a role of TORC1 in controlling the activity of eEF2K [11]. Indeed TORC1 target, S6 kinase 1, phosphorylated and deactivated eEF2K in response to insulin [24]. In contrast to these results, we reveal that dephosphorylation of eEF2 in response to TGF β is sensitive to Erk1/2 inhibition (Fig. 1 and Supplementary Fig. S3A). In fact Erk1/2 governed eEF2 dephosphorylation by inducing inactivating phosphorylation of eEF2K (Fig. 1 and Supplementary Fig. S3B).

p90Rsk consists of a family of four kinases (Rsk1-4), which share a high degree of sequence homology. Rsk1 is predominantly expressed in lung, pancreas and kidney, while Rsk2-4 are significantly expressed in the different areas in brain [19]. p90Rsk contains two functionally distinct kinase domains joined by a linker region with short N- and C-terminal tails. C-terminal kinase domain (CTKD) is homologous to the calcium-calmodulin-dependent protein kinase and causes autophosphorylation. The N-terminal kinase domain (NTKD), homologous to AGC kinase family members, is responsible for substrate phosphorylation. p90Rsk is activated in a highly coordinated manner, which is initiated by the activated Erk1/2 binding to the C-terminal tail. Phosphorylation of Ser-221 in the NTKD by the PDK1 is required for activation of p90Rsk and phosphorylation of substrates by NTKD. PDK1 is known to be activated downstream of PI 3 kinase via phosphorylation of Ser-241 in its activation loop. In mesangial cells, we have previously shown activation of PI 3 kinase by TGF β , [4]. Now we show that TGF β time-dependently increases the phosphorylation of PDK1 (Supplementary Fig. S7). These results indicate that TGF β may utilize PDK1 leading to activation of p90Rsk [19]. In addition, the hydrophobic motif autophosphorylation at Ser-380 by the CTKD is necessary for the activity of p90Rsk. In the present study, we demonstrate that TGF β increases phosphorylation of p90Rsk at Ser-380 (Fig. 2), indicating its activation. Moreover, we provide the first evidence that, in contrast to TORC1 recruitment by insulin [16,24], TGF β -induced non-Smad signaling uses activated p90Rsk to increase phosphorylation of eEF2K (Fig. 3). Our results conclusively demonstrate that p90Rsk contributes to dephosphorylation of eEF2 (Fig. 3). These data are also in agreement with the observations obtained with the G-protein coupled receptors [11]. Therefore, we conclude that TGF β receptor serine/threonine kinase utilizes non-Smad p90Rsk signaling to induce elongation phase of mRNA translation.

At the molecular level, increase in protein synthesis is required for cellular hypertrophy [7,10,25]. Role of TORC1 has been extensively studied in relation to mRNA translation initiation and elongation phases during the progression of diabetic kidney disease [25,26]. For example, we have recently reported that administration of rapamycin to mice with type 2 diabetes significantly ameliorated the renal hypertrophy [24]. Recently, p90Rsk has been shown to feed into the TORC1 signal transduction. Thus phorbol-stimulated p90Rsk phosphorylates the TSC2 protein at Ser-1798, resulting in its inactivation, leading to activation of TORC1, which regulates both initiation and elongation processes of mRNA translation [11,25–27]. Here we present results to show direct involvement of p90Rsk in TGF β -induced phosphorylation of the eEF2, which regulates the elongation of mRNA

translation. We demonstrate that p90Rsk contributes to TGF β -stimulated protein synthesis, which results in mesangial cell hypertrophy (Fig. 4). These data demonstrate that intercepting p90Rsk may provide an attractive new therapy to prevent mesangial hypertrophy during the early progression of diabetic nephropathy where TGF β acts as the pathological cytokine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

TGFβ decreases phosphorylation of eEF2 by increasing phosphorylation of eEF2K in Erk1/2-dependent manner. (A and B) Lysates of mesangial cells incubated with 2 ng/ml TGFβ for the indicated periods of time were immunoblotted with the indicated antibodies. (C and D) Mesangial cells were treated with 5 μM U0126 for 1 hour prior to incubation with TGFβ for 15 minutes. The cell lysates were immunoblotted with the indicated antibodies. The quantification of these data is provided in Supplementary Fig. S1.

Figure 2.

TGFβ stimulates p90Rsk in Erk1/2-dependent manner. (A) Lysates of mesangial cells incubated with 2 ng/ml TGFβ for the indicated periods of time were immunoblotted with the indicated antibodies. (B) Mesangial cells were treated with U0126 and TGFβ as described in Fig. 1C. The cell lysates were immunoblotted with the indicated antibodies. (C) Mesangial cells were transfected with vector or dominant negative Erk2 plasmid. Forty-eight hours post-transfection TGFβ was added for 15 minutes. Cell lysates were immunoblotted with the indicated antibodies. The quantification of these data is provided in Supplementary Fig. S4.

Figure 3.

Expression of kinase inactive p90Rsk prevents TGFβ-induced inhibition of eEF2 phosphorylation and induction of eEF2K phosphorylation. (A and B) Mesangial cells were transfected with vector or dominant negative p90Rsk expression plasmid. Forty-eight hours post-transfection, TGFβ was added for 15 minutes. Cell lysates were immunoblotted with the indicated antibodies. The quantification of these data is provided in Supplementary Fig. S6.

Figure 4.

Expression of dominant negative p90Rsk blocks TGFβ-induced protein synthesis and hypertrophy of mesangial cells. Mesangial cells were transfected with vector or dominant negative p90Rsk followed by incubation with TGFβ for 24 hours. (A) Protein synthesis was determined as ³⁵S-methionine incorporation as described in the materials and methods [7]. Mean ± SE of triplicate measurements is shown. *p < 0.05 vs control; **p < 0.05 vs TGFβ-treated by ANOVA. (B) Cellular hypertrophy was determined by measurement of total protein per cell as described in the materials and methods [7]. Mean ± SE of six measurements is shown. *p < 0.01 vs control; **p < 0.01 vs TGFβ-treated by ANOVA.